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ABSTRACT BOOK

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MAIN PROGRAM

DISRUPTION OF THE SPLICING MECHANISMS IN LEUKEMIAS

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The cellular response to environmental signals and the activation of processes such as differentiation, proliferation or cell death involves changes in gene expression. Alternative splicing modulates the expression of many genes including oncogenes and tumor-suppressor isoforms becoming a major source of diversity in the human proteome.[1]

For the majority of human genes, individual or partial exons can be included or excluded from different versions of the mature messenger RNA by alternative splicing. Many alternative splicing factors affect this process to determine which mRNA isoforms will become a templates for protein synthesis in a given cell type during a specific developmental stage.

Different splice variants of a given protein can display different or even antagonistic biological functions. The regulation of alternative splicing modulates the composition of this diversity to fulfill the physiological requirements of a cell. When control of alternative splicing is disrupted, the result can be a failure to meet cellular and tissue requirements resulting in dysfunction and disease.[2,3] Therefore, appropriate control of their synthesis is required to assure the complex orchestration of cellular processes within multicellular organisms.

Human genes express remarkably complex pre-mRNAs, containing an average of eight exons, with introns making up 90% of the transcription unit. Introns are much longer than exons and most of their nucleotide sequence seems to be irrelevant except for the so called donor and acceptor consensus sequences located at their extremities and the sequence that precedes the acceptor sequence. Introns must be excised precisely to generate bona fide mRNA molecules; otherwise translational frame shift would be introduced. The main role of the splicing machinery, called spliceosome, is to identify bone fide exons, ignore pre-mRNA segments that resemble exons (pseudoexons), join contiguous exons without inadvertent skipping and appropriately regulate alternative splicing to meet the physiological requirements of cells and tissues. The spliceosome includes nuclear ribonucleoprotein (hnRNP), SR proteins and hundreds of auxiliary proteins, committed to intron excision and exon joining.[4,5]

SR proteins are a family of RNA-binding proteins that are essential for splicing. They act at multiple steps of spliceosome assembly and function in both constitutive and regulated splicing. hnRNP proteins, which rapidly associate with nascent transcripts, and SR proteins can have antagonistic effects on the alternative splicing of particular exons in several genes.4,5 Both types of factor can bind directly to precursor (pre)-mRNA transcripts, eliciting changes in the alternative splicing of various pre-mRNA substrates in a concentration-dependent manner.

The connection between alternative splicing and cancer has now been demonstrated.[6] The most common form of splicing defects are genomic splice site point mutations.[7] Ninety nine percent of the exons are flanked by an intronic dinucleotides GT and AG at the 5' and 3' splice sites respectively and mutation of these sites usually causes exclusion of the adjacent exon or, sometimes, double exon skipping. Ore than half of all exon deletions lead to truncation of the encoded protein. Mutations in the less conserved splice site consensus away from the invariant dinucleotides lead to partial aberrant splicing often with a mild phenotype. Another way that mutations can cause aberrant splicing is by the inappropriate creation of cryptic site signals. Finally, consensus binding site for the SR proteins can be disrupted by mutations.

Another mechanism that can affect the splicing of many cellular transcripts and might account for some of the known splicing changes in cancer is represented by changes in the expression of SR and hnRNP proteins.[8,9] Some alternative splicing factors, especially from the hnRNP family, are elevated in cancer cells, and the increased expression of some of these proteins correlates with patient prognosis and can serve as a diagnostic marker for malignancy. There are at now many evidences showing that high levels of hrRNP or SR proteins are correlated with cancer.

The splicing defect can interfere with the function of different tumor suppressors, such as BRCA1/2, WT1, APC, TP73 (p73) and TSC1/2 resulting in their inactivation and consequent promotion of different types of cancer. In addition, the activities of many oncogenes are modulated by alternative splicing as well. Alternative splicing also has an important role in programmed cell death: many central components of this process are encoded by alternatively spliced mRNAs, and often the products of alternative splicing have opposing effects on apoptosis. Many other genes that are involved in proliferation and invasiveness are alternatively spliced, and specific isoforms that contribute to the transformed phenotype are frequently elevated in tumors. Finally, transcription factors, proteins involved in signal transduction such as tyrosine kinase proteins and transmembrane receptors can also be disrupted.

Targeting of alternative splicing can be used to selectively kill cancer cells. There are many examples of alternative splicing changes involved in apoptosis. BclX is a member of the Bcl2 family that has an important role in the breakdown of mitochondria during apoptosis, and it is alternatively spliced between a long antiapoptotic form (BclxL) and a short apoptosis-promoting form (BclxS), which is made by use of an upstream 5' splice site. The downstream 5' splice site of Bclx can be blocked directly by stable antisense oligonucleotides that divert splicing toward the proapoptotic upstream 5' splice site.[10] This is a particularly powerful approach, because the more antiapoptotic isoform a cancer cell has, the more cytotoxic proapoptotic form can be made by switching splice site usage. Several agents are thought to affect BclX splicing at the level of transacting splicing factors. In conclusion, there is now ample evidence that just as alternative splicing is important for differentiation, so aberrations of alternative splicing are important for cancer. Various strategies are currently being used to exploit alternative splicing for the diagnosis and treatment of cancer. Potential therapeutic targets that need additional exploration include the trans-acting factors that cause alternative splicing in cancer cells. Equally, we are at an early stage in characterizing the full repertoire of cancer-associated alternatively spliced isoforms. Whereas alternative splicing in cancer has long been recognized, its significance in the diagnosis and treatment of cancer is a recent discovery.

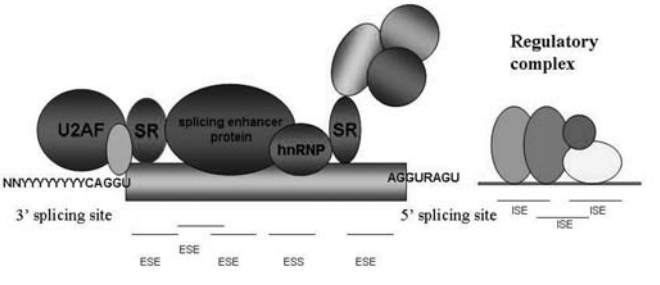

Figure 1. RNA base pairing is responsible for the exact joining of exons at the correct nucleotides. Exons and introns contain sets of enhancer and suppressor elements that refine bone fide exon recognition. Some exon splicing enhancers (ESEs) bind SR proteins and recruit and bind components of the spliceosome such as U2AF. Exon splicing suppressors (ESSs) bind protein components of heterogeneous nuclear ribonucleoproteins (hnRNP) to repress exon usage. Some intronic splicing enhancers (ISEs) bind auxiliary splicing factors that are not normally associated with the spliceosome to regulate alternative splicing.

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ROLE OF GENETICS IN THE DIAGNOSIS AND CLASSIFICATION OF T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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T-cell acute lymphoblastic leukemia (T-ALL) arises from disruption of signalling pathways that control proliferation, differentiation, and function of normal thymocytes and leads to their malignant transformation.[1] Genomic characterization of T-ALL was very limited until the development of FISH, CGH, array-CGH and PCR which revealed multiple molecular events in different pathogenic pathways. Indeed, genetic data was available for no more than 30% of T-ALL in 2004 while today the information we have in over 80% of cases indicates the pathogenesis of T-ALL is a "multi-step" process with concomitant molecular hits affecting cell proliferation, differentiation and survival.[2,3] Gene deregulation creates a defect in cell cycle control (for example *CDKN2A/CDKN2B*); impairs cell differentiation (*HOX* genes, *MLL, LYL1, TAL1/2* and *LMO1/2*); confers a proliferative and/or survival advantage (such as LCK and ABL1); and ensures self-renewal properties (*NOTCH1*) (Table 1 and 2).

In this rapidly changing scenario new genomic lesions are continually emerging. For example extensive application of array-CGH identified new recurrent chromosome duplications and losses. In 30% of pediatric T-ALL samples dup(9)(q34) varied in size but had a common duplicated region spanning *NOTCH1, MRLP41, SSNA1*, and *PHPT1* genes in all cases.[4] A stable marker, dup(9)(q34) seems to confer resistance to standard chemotherapy but can be used for monitoring minimal residual disease. The 6q23/*MYB* duplication increases MYB expression threefold as does the cryptic t(6;7)(q23;q34)/*TCRB-MYB* translocation.[5,6] Interestingly, an *in vivo* model showed MYB knockdown restored normal T cell differentiation, suggesting MYB over-expression delineates a specific oncogenic pathway in T-ALL leukemogenesis. Consequently, MYB may be worth investigating as a therapeutic target.[6]

Somatic *NF1* microdeletions that are similar to the germinal found in 510% of patients with type 1 neurofibromatosis were detected in pediatric acute myeloid leukaemia and T-ALL, with the incidence in the latter being estimated at roughly 3%.[7] In the majority of T-ALL bearing NF1 microdeletions mutational studies detected loss of function mutations of the other *NF1* allele, suggesting *NF1* acts as a tumor suppressor gene in the pathogenesis of these leukemias. Since *NF1* deficiency leads to RAS signaling pathway activation, treatment with RAS inhibitors such as farnesylthiosalicylic acid may be worth exploring.[7]

Mutational studies also identified other molecular events in T-ALL (Table 2). JAK1 gain of function mutations in 18% of T-ALL, is a new prognostic marker which is associated with poor response to therapy, a high relapse rate and short overall survival. Since all cases with JAK1 mutations also bear NOTCH1 mutations, the two aberrant pathways appear to cooperate in T-ALL pathogenesis or progression.[8]

Table 1. T-ALL: Genes, mapping, chromosome abnormalities, incidence and relevant studies.

Gene	*Locus*	*Chromosome aberration*	*Incidence*	*References*
TCRB	7q34	t(7;v)(q34;v)/*TCRB-v*	19%	Cauwelier B *et al.,* 2006
TCRAD	14q11	t(14;v)(q11;v)/*TCRAD-v*	17.4%	Cauwelier B *et al.,* 2006
TAL1	1p32	t(1;14)(p32;q11)/*TAL1-TCRAD* del(1)(p32)/*SIL-TAL1*	40%	Palomero T *et al.,* 2006
TLX3	5q35	t(5;14)(q35;q32)/*TLX3-NKX2-5* t(5;14)(q35;q11)/*TLX3-TCRD* t(5;7)(q35;q21)/*TLX3-BCL11B*	20%	Nagel S *et al.,* 2007 Su XY et al,2004
MYB	6q23	dup(6)(q22q23)/*MYB* t(6;7)(q23;q34)/*TCRB-MYB*	8.4%	Lahortiga I *et al.,* 2007 Clappier E *et al.,* 2007
CDKN2A	9p21	del(9)(p21)	80%	Bertin R *et al.,* 2003
NOTCH1	9q34	t(7;9)(q34,q34.3)/*TCRB-NOTCH1* dup(9q34)/*NOTCH1-MRLP41-SSNA1-PHPT1*	rare 30%	Reynolds TC *et al.,* 1987 Van Vlierberghe P *et al.,* 2006
ABL1	9q34	t(9;22)(q34;q11)/*NUP214-ABL1* t(9;14)(q34;q32)/*EML1-ABL1* Episomal amplification/*NUP214-ABL1*	3% rare 5%	Berger R *et al.,* 1991 De Keersmaecker K *et al.,* 2005 Graux C *et al.,* 2004
LMO2	11p13	t(11;14)(p13;q11)/*TCRAD-LMO2* del(11)(p13;p13)	5-10% 5%	Marculescu R *et al.,* 2002 Van Vlierberghe P *et al.,* 2006
LMO1	11p15	t(11;14)(p15;q11)/*LMO1-TCRD* t(7;11)(q34;p15)/*TCRB-LMO1*	9%	Valge-Archer V *et al.,* 1998
CALM	11q14	t(10;11)(q14;q23)/*CALM-AF10*	10%	Dik WA *et al.,* 2005
NUP98	11p15	t(3;11)(q12.2;p15)/*NUP98-IQCG* t(4;11)(q21;p15)/*NUP98-RAP1GDS1* t(10;11)(q25;p15)/*NUP98-ADD3* t(11;18)(p15;q21)/*NUP98-SETBP1*	rare	Pan Q *et al.,* 2007 Mecucci C *et al.,* 2001 Romana SP *et al.,* 2006 Panagopoulos I *et al.,* 2007
MLL	11q23	t(11;v)(q23;v)/*MLL-v*	5%	Soulier J *et al.,* 2005
ETV6	12p13	t(9;12)(p24;13)/*ETV6-ABL1* t(9;12)(p24;p13)/*ETV6-JAK2*	rare	Van Limbergen H *et al.,* 2001 Gloc E *et al.,* 2002
LCK	1p34	t(1;7)(p34;q34)/*TCRB-LCK*	rare	Burnett RC *et al.,* 1994
TAL2	9q32	t(7;9)(q34;q32)/*TCRB-TAL2*	rare	Marculescu R *et al.,* 2003
HOX11	10q24	t(7;10)(q34;q24)/*TCRB-HOX11* t(10;14)(q24;q11)/TCRAD-HOX11	19%	Berger R *et al.,* 2003
HOXA	7p14	inv(7)(p15q34)/*TCRB-HOXA* t(7;7)(p15;q34)/*TCRB-HOXA* t(7;14)(p15;q11)/*TCRD-HOXA*	5%	Cauwelier B *et al.,* 2007

Table 2. T-ALL: Genes, mutation mapping, incidence and relevant studies.

Gene	*Exons*	*Incidence*	*References*
NOTCH1	26(HD),27(HD),34(PEST)	50%	Weng A P *et al.,* 2004
FBW7	9-10	rare	Thompson BJ *et al.,*2007
N-RAS	1-2	4%	Kawamura M *et al.,*1998
FLT3	14(JM))	rare	Van Vlierberghe *et al.,*2005
PTEN	2-3-4-5(PD), 7(C2 D)	8%	Palomero T *et al.,*2007
JAK1	2-5(FERM), 18(KINASE)	18,4%	Flex E *et al.,*2008

Rather than investigating one specific gene, gene expression profiling (GEP) assesses over- and under- expression of many genes and serves to characterize specific sub-groups of T-ALL. Six distinct gene expression profiling signatures, often occurring in the absence of the corresponding genomic aberrations, are associated with over-expression of *LYL1, HOX11, TAL1, LMO1, LMO2,* and *HOX11L2* oncogenes, are predictive of outcome and indicate leukemic cell maturation was arrested at a specific stage of normal thymocyte development.[9] Remarkably, GEP showed diverse genetic lesions may converge on a class of oncogenes. In T-ALL the GEP signature, characterized by over-expression of a large set of HOXA cluster genes, is associated with MLL-translocations, *CALM/AF10, SET-NUP214*, and inv(7)/*TCRB-HOXA*.[10-14]

When genomic characterization was flanked by GEP to detect pathway abnormalities, some very interesting results were reported.15 PTEN loss of function mutations emerged as recurrent events that bypass the NOTCH1 signalling pathway and sustain malignant T-ALL cell growth. Indeed, PTEN mutations, when concomitant with NOTCH1 gain of

function mutations, identify a subgroup of T-ALL which does not respond to specific NOTCH1 y-secretase inhibitors.15

As we have seen the advent, and integration, of modern techniques for genomic analysis and GEP have impacted greatly on our understanding of the biological background of T-ALL. Today we stand on the threshold, ready to achieve for the first time a detailed classification of this heterogeneous disease which will serve for prognostic stratification, selection of the most appropriate therapeutic options and identification of specific lesions for monitoring minimal residual disease.

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EPIGENETIC CHANGES IN THERAPY-RELATED AML/MDS: PATHOGENETIC AND THERAPEUTIC RELEVANCE

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Epigenetic changes have been shown to play a significant role in the pathogenesis of cancer. In particular, the model of leukemogenesis in therapy-related myelodysplastic syndrome/acute myeloid leukemia (t-MDS/AML), includes hypermethylation of tumor suppressor genes as one of the leading transforming events, cooperating with genetic alterations.[1,2]

In MDS/AML, we have shown that several genes known to be inactivated in pathways of cellular transformation are characterized by aberrant promoter hypermethylation. Hypermethylation of the promoter region of the gene encoding the pro-apoptotic death-associated protein kinase 1 (DAPK1) is a frequent event in AML and in MDS, in particular in therapy-associated forms.[3] Similarly, we found a high proportion of t-MDS/AML cases to be hypermethylated for other genes involved in carcinogenesis, including the DNA repair enzymes BRCA-1[4] and rarely MGMT, the detoxification enzyme GSTP1 and the pro-angiogenic gene E-cadherin (Scardocci et al, unpublished observation).

The potential reversibility of DNA and chromatin modifications makes chromatin remodeling enzymes attractive targets for therapeutic intervention in these diseases.[5] Epigenetic treatment using 5-azacytidine (azacytidine) and 5-aza-2-deoxycytidine (decitabine), which inhibit DNA methyl-transferase (DNMT) activity, has shown to be effective in inducing remission, reducing transfusion needs, improving quality of life and prolonging survival of MDS patients.[6-8] The mechanisms of action of DNA-hypomethylating agents have not been completely elucidated, but one of the effects is induction of apoptosis. Looking at kinetics of methylation and karyotype changes during decitabine treatment in vivo, Mund et al. found that karyotype normalization precedes global DNA demethylation, indicating that the primary effect of this drug might be cytotoxicity against the abnormal clone.[9] Using the HL-60 cell line as a model, non-dose-dependent global DNA hypomethylation and apoptosis were observed following 5-azacytidine treatment.[10] DNA hypomethylation was in particular evident in the sorted apoptotic cell fraction (41% decrease with 1 microM after 24 hours), while non-apoptotic cells retained a methylation pattern similar to untreated cells (+/- 6%), indicating that apoptosis may be a specific consequence of methylation reversal.[10] In this line, using RT-PCR arrays, we observed a significant, more than 3-fold upregulation in the expression of 12 apoptosis genes in HL-60 cells following decitabine treatment, while expression of 57 apoptosis genes did not significantly change. DAP-kinase 1 was one of the genes showing strong upregulation, in particular in apoptotic cells, sorted using Annexin-5-conjugated immmunomagnetic beads. However, silencing of DAPK1 protein expression by siRNA did not rescue HL60 cells from apoptosis, suggesting that other pathways are also crucial for induction of apoptosis by decitabine. Other genes, which resulted to be upregulated by dectabine in our PCR array, belonged to the TNF family (CD40, CD70), or were apoptosis facilitators, as BCL2-L10. Further studies are needed to elucidate whether the crucial player for induction of apoptosis by hypomethylating agents can be identified among these candidates.

Treatment with azacytidine and decitabine also induces global DNA hypomethylation in vivo, but no associations between specific methylation markers and their changes during treatment and response have been observed so far.[11,12] Other markers for predicting response to hypomethylating drugs are needed. Chromosome 7 abnormalities, which are often found in therapy-related MDS/AML, were frequently present in complete responders to azacytidine.[7, 8, 13, 14] This raises the question whether cells carrying this chromosomal abnormality exhibit higher susceptibility to hypomethylating treatment.

In addition to DNA methylation, epigenetic regulation of gene expression involves also histone methylation and acetylation. The latter is controlled by histone acetyl-transferase (HAT) and histone-deacetylase (HDAC). Inhibition of HDAC activity restores a permissive gene expression state. The understanding of epigenetic regulation of gene expression has led to rational combination therapies adding a HDAC inhibitor to a hypomethylating agent.[11, 12] These combinations have been shown to have synergistic activity on gene reactivation and leukaemia cell kill. In this line, the MDS committee of the GIMEMA acute leukemia-working party conducted a phase II study on the combination of the DNMT inhibitor 5-azacytidine, the histone deacetylase inhibitor valproic acid (VPA), and all-trans retinoic acid (ATRA) in patients with intermediate-2/high-risk myelodysplastic syndromes. The protocol included 62 patients (43 males, 19 females, median age 67 years, range 53-83 yrs), with RAEB (37 patients), RAEB-t (21 pts), and CMML (4 pts). Treatment was feasible, with about 60% overall survival at 1 year in this poor prognosis MDS patient group.

Studies of methylation profiles at diagnosis and following epigenetic treatments are needed to identify new biomarkers associated to response. Furthermore the definition of the genetic background of the host, including polymorphisms of enzymes involved in detoxification and drug metabolism might help to identify new predictive factors associated to treatment response. The ultimate goal is to elaborate patient-tailored approaches.

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IMMUNOSUPPRESSIVE ACTIVITY OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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Bone marrow (BM) mesenchymal stem cells (MSCs) are multipotent non-hematopoietic progenitor cells that can differentiate into BM stromal cells, osteoblasts, adipocytes, chondrocytes, tenocytes, skeletal myocytes, neurons, and cells of visceral mesoderm. They typically do not express hematopoietic SC (HSC) markers, but a quite specific pattern of molecules, such as SH2 (CD105), SH3 and SH4 (CD73), CD106 (VCAM-1), CD54 (ICAM-1), CD44, CD90, CD29 and STRO-1.

In the last few years, it has become clear that MSCs also possess immunoregulatory properties, which have been extensively studied and characterized for their relevance in immune responses, as well as their potential usefulness in BM transplants. Mouse BM-derived MSCs can dramatically down-regulate the response of naïve and memory antigen-specific T cells to their cognate peptide, and this effect is primarily cell contact-dependent.[1] By contrast, human MSCs may influence different effector cells, including $CD4^+$ and $CD8^+$ T cells,[2] NK cells[2,3] B cells,[2,4] monocytes and dendritic cells (DC)[5] and their effect seems to be mainly depending upon the release of soluble factors, such as transforming growth factor (TGF)-β1, hepatocyte growth factor (HGF)[3] prostaglandin (PG)E2,[3] indoleamine 2,3-dioxygenase (IDO),[2] and interferon-gamma (IFN-γ).[2] The primary mechanisms involved in the MSC mediated suppressive activity on immune effector cells, as well as the role of MSC-derived stromal cells in normal lymphoid development are still partially unknown. However, it has been shown that MSC infusion significantly prolongs the survival of MHC-mismatched skin grafts in baboons,[6] reduces the incidence of graft-versus-host disease (GvHD) after allogeneic HSC transplantation in humans, and treats severe acute GvHD refractory to conventional immunosuppressive therapy.[7]

Toll-like receptors (TLRs), which are broadly distributed on cells throughout the immune system,[8] are the best studied immune sensors of invading microbes and their activation is essential for inducing the immune response and enhancing adaptive immunity against pathogens.[9] Members of the TLR family are also involved in the pathogenesis of autoimmune, chronic inflammatory, and infectious diseases.[9] Thirteen TLR1 analogues have been identified (10 in humans and 13 in mice) that recognize a wide variety of pathogen-associated molecular patterns (PAMPs) in bacteria, viruses, and fungi, as well as certain host-derived molecules.[9] Recently, a novel non-immune role for TLRs has been reported, which concerns on the maintenance of epithelial homeostasis through proliferation and tissue repair after direct injury to the epithelium[10] and stimulation of cell-cycle entry and progression in fibroblasts.[11] Moreover, it has been recently shown that the specific, ligand-mediated triggering of some TLRs, which are expressed by both murine and human MSCs, may control their proliferation and differentiation.[12, 13]

The discovery of TLR expression by MSCs prompted us to investigate the potential link between TLR signalling and the MSC-mediated immunoregulatory functions. Thus, we demonstrated that human BM-derived MSCs express high levels of TLR3 and TLR4, low levels of TLR1, TLR2, TLR5 and TLR6, whereas they do not express TLR7, TLR8, TLR9 and TLR10. Accordingly, LPS and poly I:C but not CpG ODN and R848, were able to induce NFkB activation in MSCs, as well as cytokine and chemokine production by these cells. Flow-cytometric analysis of MSCs revealed no differences in the expression of CD34, CD80, CD86, CD105 and CD106, between untreated and LPS- or poly I:C-treated cells, as well no influence on their differentiation potential, inasmuch as they retained their ability to differentiate toward osteoblasts, condrocytes and adipocytes. However, we showed that the addition in culture of LPS or poly I:C, but not of CpG ODN or R848, could reduce significantly the suppressive activity of MSCs on T cell proliferation. TLR ligation on MSCs did not influence at least some of the mechanisms that have been described to be responsible for the immunosuppressive activity of MSC on T cells so far, such as IDO activity or PGE2 production. On the contrary, TLR ligation on MSCs influence a previously unknown immunosuppressive mechanism based on Notch receptor signalling on T cells. Members of the Notch family of transmembrane receptors are critically involved in the control of differentiation, proliferation and apoptosis in several cells types.[14] Previous reports have shown that the over-expression of the Notch ligand, Jagged-1, on DCs can modulate the differentiation of T helper lymphocytes and that ligation of TLR on DCs induces a dramatic down-regulation of Jagged-1 and the over expression of the ther Notch ligand Delta-4 that possess stimulatory activity on T cells.[15] Based on this finding, we first hypothesized that Notch signalling could be involved in the suppressive activity of MSCs on T-cell proliferation. Notch signalling is mediated by the γ-secretase-mediated cleavage of the Notch receptor intracellular domain (NICD), translocation into the nucleus, and activation of CBF-1/RBP-Jκ. In agreement with our hypothesis, the immunosuppressive effect on T-cell proliferation was inhibited by both an anti-Jagged-1 neutralizing Ab and the γ-secretase inhibitor DAPT. In addition, we found that MSCs constitutively expressed Jagged-1, but not the other Notch ligand, Delta-4, and that the expression of Jagged-1 by MSCs was inhibited at both mRNA and protein levels by ligation of either TLR3 or TLR4. Taken together, these data strongly suggest that ligation of TLR3 or TLR4 on MSCs inhibits their suppressive effect on T-cell proliferation, by hampering their Jagged-1 expression and, therefore, impairing its signalling to Notch receptor expressed on T cells. Thus, we have not only identified a new mechanism responsible for the cell contact-dependent, immunosuppressive effects of MSCs on T cells, but we have also provided the first evidence for TLR involvement in such a immunomodulatory activity.

The demonstration that the immunosuppressive activity of MSCs is reduced following triggering of the TLRs that they express, suggests that infectious agents expressing PAMPs for TLR3, TLR4, but probably also for TLR2 and TLR6, should be considered at particularly high risk during this type of treatment (Figure 1).

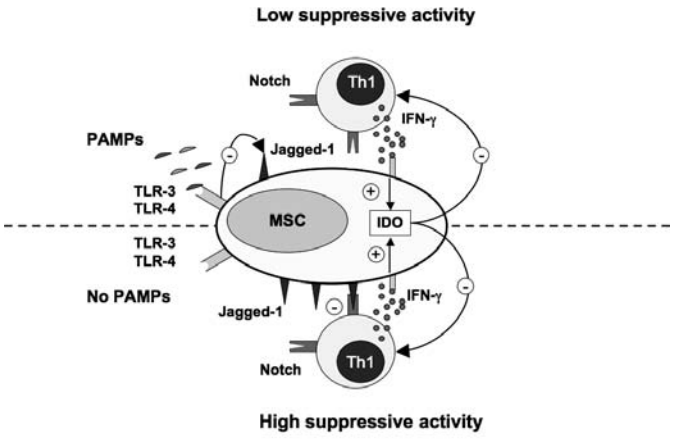

Figure 1.

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ROLE OF MESENCHYMAL CELL POPULATION IN CANCER DEVELOPMENT

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The tumorigenic potential of mesenchymal stem cell (MSC) infusion and the MSC-mediated support of developing cancers by preventing specific anti-cancer immune responses are still open questions. *In vitro* cultured human BM-derived MSCs show normal karyotype before transplantation into the patients,[1] they are not susceptible to malignant transformation after long-term in vitro culture until senescence or passage 25, and they do not exhibit telomere maintenance mechanisms.[2] However, MSCs coinfused with BM into irradiated allogeneic recipients mice may develop sarcoma and display cytogenetic abnormalities.[3] MSCs are capable of homing to the sites of injury and they may therefore provide site-specific and local immune regulation. However, MSCs may also promote tumour growth and prevent the rejection of allogeneic tumour cells.[4-13] MSCs, infused either systemically or subcutaneously inside growing B16 melanoma cells, determine the enhancement of tumour formation.[13] MSCs within tumour stroma favour breast cancer metastases in mice bearing subcutaneously MCF7/Ras or MDA-MB-231 human breast cancer xenografts.[8] *In vitro* studies suggest that molecules belonging to epidermal growth factor family may play a role in the expansion and differentiation of stromal cell precursors inside tumours.[14] More importantly, MSCs may inhibit in vitro and *in vivo* the specific anti-tumour immune response against Sp6 plasmacytoma in Balb/c mice previously immunized and refractory to tumour development.[5] On the other hand, some studies have shown that MSCs may inhibit tumour growth in mouse[13] and rat[9,10] models. Similarly, human MSCs exhibit a dose-dependent antiproliferative activity on different tumour cells lines of hematopoietic and non-hematopoietic origin, producing the transient arrest of tumour cells in the G1 phase of cell cycle, which disappears after MSC removal.[11] However, when tumour cells are co-injected with MSCs into NOD-SCID mice, tumour engraftment and growth are favoured.[11] Therefore, the clinical use of large doses of MSCs must always consider the potential side effects in terms of tumour development. However, because of their preferential migration to sites of tumour growth, MSCs may be used as vehicles for specific and precise anti-cancer drugs delivery, such as interferon-beta[7,9] and NK412, with a low number of side effects.

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REGULAMENTATIONS OF THE CELL THERAPY PROTOCOLS: THE EXAMPLE OF BERGAMO

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According to the European and Italian Laws, cell therapy products for somatic cell therapy protocols have to be produced in agreement with Good Manufacturing Practices (GMP) rules. Moreover, the Cell Factory in which the cell manipulation takes place has to be inspected and approved by the National Authorities, in our case the Agenzia Italiana del Farmaco (AIFA). Moreover, the specific cell therapy product and related clinical protocol has to be independently approved by the Istituto Superiore di Sanità.

We have received three consecutive inspections by the AIFA personnel, finally receiving formal approval of the Cell Factory in late July 2007 for the production of small injectable sterile products. In this factory we can produce a) Cytokine Induced Killer (CIK) cells, having received authorisation by ISS for a phase I study. The study was referring to the use of donor derived CIK cells for the treatment of the leukemia patients experiencing relapse following allogeneic bone marrow transplantation. The phase I study was conducted and published and the results have encouraged to proceed for a phase II study of efficacy. The protocol for this phase II study is at the moment at the AIFA agency for further specific approval. b) We were authorized to produce anti CMV specific T lymphocytes (CTL) for the treatment of CMV reactivation in severely immunosuppressed allogeneic bone marrow transplanted patients. This protocol was shared with the Cell Factory located at S. Gerardo Hospital (Monza) and is actually conducted in that site only. c) Finally, we received authorisation by ISS for a cell therapy programme in kidney transplantation, by the administation of donor's mesenchymal stromal cells (MSC) to try and ameliorate the immunosuppressive regimen. The production of MSC benefits greatly of the abrogation of Ficoll, the reduction of trypsin to a single step, the seeding of cells at very low concentrations and the avoidance of the fetal calf serum substituted by the clinical grade platelets lysate available from the Transfusion Center of the Hospital. The validation of the production protocol in two independent cell factories has been submitted for publication. The same protocol will be used for the production of MSC from healthy donors to be used for a different clinical phase II study in steroid resistant severe aGVHD bone marrow transplanted patients. The protocol has been sent for approval to AIFA.

The Cell Factory was build by the money collected by the no profit private organization Associazione Paolo Belli in Bergamo and is at the moment run by a team of persons payed in part by the Ospedali Riuniti di Bergamo and in part by the Associazione Paolo Belli. In addition to the Qualified Person (regularly authorized by AIFA and CUN), the Quality Control Responsible and the Quality Assurance Responsible, the personnel includes 3 operators for the production and 3 operators for the quality controls.

The Laboratorio di terapia cellulare "G. Lanzani" of the Hematology Unit at the Ospedali Riuniti di Bergamo represents one of the three public cell factories so far approved in Italy by AIFA and its pilot experience may help other centers in Italy and Europe to try and solve the complex problems of the industrial GMP productions when conducted inside a clinical, public, no profit organization.

INTRODUCTION TO THE LECTURE. VIRAL INFECTIONS, ANTIGENIC STIMULATION AND LYMPHOMAGENESIS

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In several lymphoid disorders there is compelling evidence that B-lymphocytes are able to recognize different extrinsic antigens and that this condition may provide a stimulus for the proliferation and survival of the lymphoma cells. When the antigenic stimulation is removed, B-cells can enter a quiescent state (memory B-cells); alternatively, if the antigenic stimulation persist, these cells may proliferate and accumulate genetic changes becoming malignant in the long run. Several viruses have been associated with lymphomagenesis: f.i. EB virus in Burkitt's lymphoma, in some T and NK-lymphomas, in 30-40% of Hodgkin's disease and in lymphomas originating in immunosuppressed patients. HTCL- Virus- 1 and HHV8 (human herpes virus 8) genomes have been demonstrated in some cells of lymphomas. Further a strict connection between HVC and lymphomas has been suggested in several patients.

Balanced chromosomal translocations involving the immunoglobulin genes and a proto-oncogene constitute a hallmark of different lymphomas (e.g. bcl-1/Ig in mantle cell lymphoma, bcl-2/Ig in follicular lymphoma, c-myc/Ig in Burkitt's lymphoma). Often, in these conditions, the translocated partner gene may interact with an active Ig locus giving rise to a dysregulated constitutive expression. Several molecular mechanisms involved in these different conditions have been proposed.

The bcl-2/Ig translocation is one of the most important examples of this condition. The pathogenesis of NHL with the t(14;18) translocation recognizes three main sequential events: a) the chromosomal translocation due to an error during the assembly of the variable region of the Ig heavy chain gene; b) the clonal expansion in the follicle center microenvironment; c) additional genetic alterations with deregulated cell growth.

However, since most B-lymphomas arise in absence of any known antigenic exposure, novel mechanisms involved in the lymphomagenesis should be sought.

Other mechanisms may become important such as the participation of the microenvironment , stimulation of B cells by idiotype specific T-cells, inhibition of apoptosis or induction in some patients of a constitutive expression of bcl-6 gene leading the cells to proliferate and preventing them to enter a resting condition.

VIRAL INFECTIONS, ANTIGENIC STIMULATION AND LYMPHOPROLIFERATIONS

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Bacterial infections are well known to be involved in lymphoproliferations, by providing an antigenic stimulation. Do viral infections behave like bacterial infections? In mucosa associated lymphoid tissue (MALT) lymphoma, which is a classical model of antigenic (bacterial) stimulation-related lymphoproliferation, Helicobacter pylori (HP) is not the only recognized infectious agent. Anti-hepatitis C virus (HCV) antibodies and viral sequences have been detected in 8 of 16 MALT lymphomas.[1] In one HP positive gastric MALT lymphoma resistant to eradication, HCV infection has been demonstrated in gastric epithelial cells. A few evidences suggest that HCV may be one of the viruses which may behave like bacteria (Table 1). First, epidemiologic studies have shown an association between HCV and lymphomas. HCV is associated with certain histologic B-cell non Hodgkin lymphoma (NHL) subtypes (marginal zone, diffuse large cell lymphomas) in endemic areas (20-40% in Italy and Japan; less than 5% elsewhere in Europe and North America).[2,3] Higher anti-HCV antibodies and HCV RNA sequences may be detected in B-NHL than in other lymphoid neoplasias or in age-matched healthy subjects.[2,3] HCV infection often precedes by years the occurrence of lymphomas. However, the classical virologic criteria necessary to establish a causal relationship between a virus and a tumor have not been fulfilled in the case of HCV and lymphoma. Relevant to this the absence of in vitro model of HCV transformation and the absence of animal model of HCV transformation argue against an orthodox lymphomagenetic role of HCV. Furthermore, neither HCV RNA nor proteins have been demonstrated in lymphomatous cells, with only few exceptions. Thus, the pathophysiology of HCV- induced B cell proliferation essentially differs from that of the herpesvirus-induced B cell proliferations by involving three main scenarios: 1) the effect of chronic HCV antigenic stimulation; 2) the effect of some HCV proteins which may provide support for B cell clonal expansion; 3) the effect of the B cell regulatory control dysfunction which accompanies HCV related clonal expansion.[4] Studies identified the specific VH and VL gene family involved, with VH-1-69 and VK A27 being the most commonly used in MC type II and malignant lymphomas.[4,5] Sequencing of these Ig variable regions has revealed that they are the product of somatic hypermutation, a trademark of maturation under antigenic stimulation.. HCV-E2 envelope glycoprotein is a possible candidate antigen, based on the finding that anti-HCV E2 B cell clones from HCV infected patients use VH1-69 preferentially and that the BCR isolated from a lymphoma in an HCV infected patient effectively binds HCV-E2. In B cells CD81 associates with the CD19/CD21 co-receptor complex.[4] The co-engagement of the BCR and the CD19/CD21/CD81 complexes lowers the threshold of B cell activation and results in the prolonged association of the BCR with lipid rafts and prolonged signalling from the rafts as compared to that achieved by BCR cross-linking alone. Thus, HCV, by virtue of binding directly to CD81 could react with a specific BCR and deliver a dual acti-

vation signal to specific B cells. Moreover, acute and chronic HCV infection causes a 5- to 10-fold increase in mutation frequency in Ig heavy chain, BCL6, p53 and Beta-catenin genes of *in vitro* HCV infected B cell lines, HCV associated peripheral blood mononuclear cells and lymphomas, by inducing double strand breaks, the repair process of which may be associated with the introduction of mutations. Mutations occur while homologous recombination repairs double strand or single strand DNA breaks, possibly mediated by error-prone DNA polymerases, and HCV activates error prone DNA polymerases. Activation induced cytidine deaminase (AID) plays a role in the hypermutation of Ig, probably by deaminating dC, and induces hypermutation in actively transcribed genes with a strong bias toward dGdC base pairs, and HCV activates the expression of AID.[4,6] Finally, B lymphocyte stimulator (BLyS) is increased in HCV infected and MC type II patients and its overexpression may contribute to B-cell clone expansions and autoantibody production.[4] Antiviral therapy against HCV-related MC type II and HCV-related lymphomas may be effective.[6] The gamma-herpesviruses, namely, the Epstein-Barr virus (EBV) and the human herpesvirus-8 (HHV-8), behave unlike bacteria and have a direct lymphomagenetic effect, by exploiting (EBV) or skipping (HHV-8) the B cell developmental pathway and by affecting specific pathways of B cell proliferation, such as the cell cycle control (p53, Rb) and Notch and Wnt signaling.[7-10] Is there any role for antigenic stimulation and for cooperation of herpesviruses with other pathogens? Plasmodium Falciparum malaria has been shown to directly induce EBV reactivation in latency-infected memory B cells.[11] If the c-myc translocation is the unifying defect of Burkitt's lymphoma (BL), how might it arise as a frequent consequence of EBV and malaria interplay? Mice expressing a constitutively active form of AID spontaneously develop c-myc translocations into the immunoglobulin locus.[11] The combination of EBV and malaria should then dramatically increase the frequency of AID dysregulation, resulting in endemic BL.[11] This scenario is highly plausible, because: (a) both EBV and malaria act as potent stimulators of B-cell proliferation, and (b) EBV is known to turn on AID.[11]Is there any role for betha-herpesviruses in lymphoproliferations? The role for cytomegalovirus in mycosis fungoides(MF)/Sezary syndrome is controversial.[12] Various studies have suggested an association between human herpesvirus-6 (HHV-6) infection and human lymphomas.[13] However, the absence of viral antigen expression in tumor cells,[14] and the finding of viral integration in normal cells[15] have ruled out a major orthodox role for HHV-6 in human lymphomagenesis. Moreover, our group has described for the first time HHV-6 infection of follicular dendritic cells (FDC) in Rosai-Dorfman[14] disease and also identified HHV-6 by immunohistochemistry in FDC in two cases of angioimmunoblastic T cell lymphoma (AITL), both following HHV-6 primary infection. The peri-follicular localisation and expression of CD10, bcl-6, and CXCL,[13] by the neoplastic cells in AITL, suggests the tumor may originate from follicle centre T cells. The survival and proliferation of these T cells is regulated by interactions with FDC, and FDC expansion is a key feature of AITL. Thus, the possibility is raised that HHV-6 might modulate the neoplastic T cells in AITL by affecting the tumor-associated FDC.

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Table 1. Associations between viruses and lymphoproliferations.

HTLV-I	in endemic areas associated with: · adult T cell leukemia, · a form of cutaneous T cell leukemia/lymphoma.
EBV	· with Burkitt's lymphoma · Hodgkin's disease (mixed type) · T cell lymphomas (angioimmunoblastic, angiocentric, and nasal lymphoma and large granular lymphocyte leukemias from Asian countries) · Malignant lymphomas in boys with X-linked lymphoproliferative syndrome · Primary cerebral high grade B cell non Hodgkin's lymphomas (100%) and some systemic lymphomas of the immunoblastic or large cell type in HIV-1 infected patients · Post-transplant lymphoproliferative disorders in transplant patients.
HHV-6	· Rosai Dorfman disease?
HHV-8	· Primary effusion lymphoma (PEL) · Multicentric Castleman disease of plasma cell type/plasmablastic lymphoma · Solid B- non Hodgkin lymphoma (PEL variant) · Germinotropic Lymphoproliferative Disorder · Common variable immunodeficiency?
HCV	· B- cell non Hodgkin lymphoma (marginal zone and diffuse large cell types)
SV40	· B-cell non Hodgkin lymphoma?

GENE EXPRESSION PROFILING: WHAT DOES IT OFFER IN LEUKEMIA COMPREHENSION?

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Gene expression profiling has represented, over the last decade, a revolutionary tool that has allowed to gain fundamental insights in disease pathogenesis: this is mostly true in onco-hematology, since the analysis of neoplastic cells is relatively simple because of their easy accessibility.

We can nowadays refer to two different gene expression profiling eras: in the past, this technique was used as a classification device, whereas it is presently used for other functional purposes, such as integration with other genetic techniques (i.e. array CGH, SNP and copy number analysis - CNA - mutational analysis and methylation analysis) that ultimately permit an in-depth characterization of the events that lead to malignant transformation, disease prognostication and outcome prediction and, wherever possible, pathway discovery.

Identification of profiles associated with known genetic lesions. During the first era, it was first shown that acute myeloid leukemia (AML) was simply discernible from acute lymphoblastic leukemia (ALL);[1] within ALL, it was possible to identify specific signatures that allow to discriminate the different molecular aberrations (namely, ALL1/AF4, TEL/AML1, E2A/PBX and BCR/ABL), lineage derivation (T-lineage ALL vs B-lineage ALL),[2,3] and the various maturative stages, such as, in T-ALL patients, immature vs cortical vs post-thymic T-cell development;[4] in T-ALL, it

was also shown that a small set of genes is predictive of chemoresistance and long-term outcome.[5] Furthermore, it has been possible to identify some genes that are specifically expressed in certain subgroups, and may be used as therapeutic targets: this is case of FLT3 overexpression in MLL-rearranged leukemias.[6]

Similarly, gene expression profiling has allowed to distinguish diverse AML subtypes with some evidence of easy distinction, as t(8;21)/AML-ETO, t(15;17)/PML-RARα and inv(16)/CBFB-MYH11.[7] Different results were obtained when evaluating the gene expression profiling of chronic lymphocytic leukemia (CLL): in fact, while it was clearly highlighted that CLL is different from other lymphoproliferative disorders, it has also been shown that CLL is a single entity and that the IgVH mutational status does not affect CLL gene expression, though a limited number of genes is differentially expressed between mutated and unmutated cases.[8,9] Overall, these results opened the way to the understanding that gene expression profiling may be used in a diagnostic setting: as a matter of fact, Haferlach[10] and colleagues showed that by gene profiling it is possible to reach a 95% correct diagnostic prediction, when analyzing roughly 1000 individuals with oncohematologic disorders. Noteworthy, some leukemia subtypes are more difficult to be classified by gene expression profiling and, in particular, two representative examples are provided by ALL with and/or without t(9;22) and myelodisplastic syndromes (MDS): in both instances, the fact that these cases may be misclassified points to the heterogeneity of these diseases and, possibly, to the presence of other subentities previously unidentified.

Identification of profiles associated with novel genetic lesions. In the second era of gene expression profiling, the use of this technique has slightly changed mostly because of its integration with other approaches. Several examples in this respect are provided by AML and T-lineage ALL, where biologic progress has been striking.

In AML, the first integration came from the identification of different profiles associated with 1) FLT3 mutation, 2) MLL partial tandem duplication (MLL-PTD), 3) NPM1 mutations.

1) Within FLT3 mutations, it has been possible to correlate different gene expression signatures with the presence of either FLT3 internal tandem duplication (FLT3-ITD) or FLT3 tyrosine kinase mutations (FLT3-TKD), thus defining a small set of genes that are predictive of the two mutations with 100% accuracy, and providing evidences that different pathways (cell cycle, signal transduction and transcription) are deregulated by the FLT3-ITD or FLT3-TKD.[11]

2) As for MLL-PTD, it was not possible to identify a clear and reproducible signature: this finding indicates that this aberration does not define a specific and univocal subtype and does not deregulate a specific pathway.[12]

3) Evaluation of NPM1 mutations led to shown that this mutation is most frequently correlated with a gene expression profile characterized by an involvement of genes related to signaling and apoptosis, as well as genes belonging to the HOX family, comprising both HOXA and HOXB members, thus indicating that also NPM1 mutations act by deregulating the HOX pathway (see also below).[13]

In T-ALL, the biologic knowledge was previously scarce; at present, novel lesions are emerging and they include rearrangements (for example SIL/TAL1, CALM/AF10, and NUP214/ABL), gene overexpression (i.e. LYL, TLX1, TLX3, TAL1) and mutations (NOTCH1, PTEN and JAK1). In this changing context, gene expression profiling has helped to understand which pathway/s is specifically deregulated. Among the most interesting findings, it is noteworthy to remark that only by applying gene expression profiling studies it has been possible to understand that several lesions, namely CALM/AF10, TCR rearrangements, MLL rearrangements, SET-CAN rearrangements and inv(7), all contribute to a deregulation of the HOX genes and, therefore, to a development defect.[14-17]

Similarly, JAK1 mutations contribute to defects in the STAT signaling pathway. In both cases, gene profiling allowed a precise pathway discovery, which may ultimately lead to define new therapeutic strategies.[18]

Finally, another piece of information that derives from the use of gene expression profile studies is the identification of novel leukemic entities, with "mixed" features: in fact, Wouters and colleagues[19] have recently described a novel subgroup of AML that is characterized by the concomitant expression of myeloid and T-lineage genes. This subgroup of patients appears to be characterized by an hypermethylation of the CEBPA promoter as well as by the presence of NOTCH1 mutations. This finding has also a clinical impact, since these patients seem to have an inferior outcome and may indeed benefit from a different chemotherapeutic approach. Interestingly, in T-ALL we recently identified a small subgroup of patients who express myeloid genes, and are characterized by high levels of CEPBA expression, suggesting an opposed behavior to that described by Wouters et al.[19]

Conclusions. In conclusion, gene expression profiling in hematology has offered a new classification tool, has permitted the identification of potential therapeutic targets and is also offering hints on the deregulation of certain cellular pathways in a given molecular aberration, prompting to define tailored therapies.

Integration with microRNA profiling may indeed lead to unravel novel mechanisms of transformation and specific interaction between these class of small molecules and their gene targets.

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GENE EXPRESSION PROFILING OF LYMPHOMAS: A NEW WAY TO UNDERSTAND, CLASSIFY AND CURE

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The practice of clinical medicine and the process of biomedical research have been transformed by the decoding of the human genome. The use of DNA microarrays to find gene expression patterns in disease and biological processes has already begun to have a significant impact on modern medicine. The study of hematological malignancies has especially benefited from gene expression profiling (GEP), including discoveries about histogenesis, molecular pathogenesis, classification, and prognosis. In particular, GEP is a powerful tool to uncover complex molecular networks in cancer and, specifically, in malignant lymphomas, which will hopefully lead, in the future, to more efficacious choice of novel therapeutic approaches. As to what B-cell derived non Hodgkin lymphomas (B-NHL) are concerned, several studies have been conduced on diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), and Burkitt's lymphoma (BL). Briefly, within DLBCL, two major molecular subtypes, the activated B-cell-like (ABC) and the germinal center B-cell-like (GCB) DLBCL, have been defined.[1,2] Compared to GCB DLBCL, ABC DLBCL showed a vast difference in gene expression and constitutive expression of NFκB and its target genes. In retrospective analyses, the molecular phenotype of ABC DLBCL was associated with inferior survival.[3] Furthermore, GEP allowed the molecular separation of BL from DLBCL and revealed a Burkitt-specific signature which was also expressed by a subset of tumors currently classified as DLBCL.[4, 5] Of note, whether patients with a DLBCL displaying a Burkitt-specific gene expression signature may benefit from alternative therapeutic approaches will have to be determined in future prospective clinical trials.

In FL, two outcome-related signatures, termed Immune response 1 (IR1) and Immune response 2 (IR2), have been identified by GEP, indicating a significant role of the microenvironment in tumor development and progression.[6] IR1, composed of genes mostly expressed by T-cells, was found to be associated with a more favorable clinical course, and IR2, enriched for genes expressed by macrophages and follicular dendritic cells, was found to be associated with an inferior clinical course.[6] More recently, GEP showed that grade I to IIIa FLs had a relatively homogeneous molecular profile, irrespectively of the histological grade.[7] In addition, grade IIIb FL has been shown to be actually more related to FLs rather than to DLBCLs providing a molecular rationale for the new upcoming WHO Classification in this setting.[7]

In MCL, a gene expression-based proliferation signature of 20 different genes was identified that is able to predict survival of MCL patients in a linear fashion.[8]

More recently, GEP was used to better elucidate the molecular basis of peripheral T-cell lymphomas (PTCLs), and, first of all, PTCL not otherwise specified (PTCL/NOS) have been investigated. Briefly, It was shown that PTCL/NOS could be divided into two different subgroups based on the expression of NF-kappaB related genes. One-third of PTCL showed clearly reduced expression of NF-kappaB genes, while the other group was characterized by higher expression of these genes. Of interest, the expression profile associated to reduced expression of NF-kappaB genes was significantly associated with shorter survival of patients.[9, 10] In a second study a possible third group was identified. However, it is possible that such distinction possibly reflected, at least in part, the presence of reactive components in the PTCL samples.[11] Interestingly, it was showed that PTCLs/NOS are most closely related to activated peripheral T lymphocytes, either $CD4^+$ or $CD8^+$, basing on the GEP.[12] In addition, PTCLs/NOS displayed deregulation of relevant functional cell programs. In particular, among others, *PDGFRA*, a gene encoding for a tyrosine-kinase receptor, turned out to be aberrantly expressed by PTCL/NOS.[12] Notably, both phosphorylation of PDGFRA and sensitivity of cultured PTCL cells to imatinib were demonstrated. In addition, it was demonstrated that PTCL/NOS often present with aberrant phenotype.[12] Of note, this may have relevant therapeutic consequences as, for example, CD52 and CD4 (against which monoclonal antibodies have been developed) are frequently defective in these tumors.[13] Notably, over-expression of genes belonging to a "proliferation signature" was associated with significantly shorter patients survival, confirming a previous observation that Ki67 expression on routine diagnostic material can be relevant for prognostic stratification.[14,15]

Subsequently, angioimmunoblastic lymphomas (AILT) was studied by GEP. It was reported that AILT and PTCL/NOS had rather similar GEP, possibly sharing common oncogenic pathways. In addition, it was found that the molecular signature of follicular T helper cells was significantly over-expressed in AILT. Finally, several genes deregulated in AILT, representing potential therapeutic targets such as *PDGFRA* and *VEGF*, were identified.[16,17] Finally, as to what anaplastic large cell lymphomas (ALCLs) is concerned, it was shown that these tumors could be divided in two clusters, corresponding essentially to morphologic subgroups and clinical variables. Interestingly, supervised analysis showed that ALK+ALCL and ALK- ALCL have different GEPs, further confirming that they are different entities.[18] In conclusion, while GEP led to important discoveries in lymphomas patho-biology, there are many questions left to study and the translation of these tools and their results into the clinic has just begun.

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INTEGRATIVE GENOMIC APPROACH TO THE MOLECULAR BIOLOGY OF MULTIPLE MYELOMA

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Multiple myeloma (MM) is a disease of antibody-secreting bone marrow plasma cells that accounts for 10% of all hematological malignancies. The broad clinical spectrum of plasma cell dyscrasias range from a pre-malignant condition termed monoclonal gammopathy of undetermined significance (MGUS) to smouldering MM (SMM), truly overt and symptomatic MM, and extra-medullary myeloma/plasma cell leukemia (PCL).[1,2] MM is characterised by a profound genomic instability that involves both ploidy and structural rearrangements.[3] Nearly half of MM tumours are hyperdiploid (H-MM) characterised by recurrent trisomies, particularly of chromosomes 3, 5, 7, 9, 11, 15, 19 and 21; the non-hyperdiploid (NH-MM) tumours are cases with a hypodiploid, pseudodiploid or near tetraploid chromosome number, and are frequently associated with chromosome 13 deletions and immunoglobulin heavy chain (IGH) locus translocations involving a promiscuous number of partner loci, mainly CCND1 (11q13), FGFR3/MMSET(4p16.3), MAF (16q24), MAFB (20q) or CCND3 (6q).[4-7] It has also been demonstrated that almost all MM patients are affected by deregulation of one of the cyclin D genes (CCND1, 2 or 3), which may therefore play an important role in the molecular pathogenesis of the disease.

MM patients can be stratified into five molecular groups on the basis of the presence of known IGH translocations and cyclin D deregulation (TC classification): TC1 is characterised by t(11;14) or t(6;14); TC2 is associated with hyperdiploidy and low-moderate levels of CCND1 in the absence of IGH translocations; TC3 includes tumours not falling into any of the other groups, most of which express CCND2; TC4 is associated with t(4;14) and high CCND2 levels; and TC5 expresses the highest levels of CCND2 in association with either t(14;16) or t(14;20).[8,9]

We have combined fluorescence in situ hybridization (FISH) analyses and global gene expression profiling (GEP) in a series of studies aimed at elucidating the transcriptional profiles associated with plasma cell dyscrasias in a panel of newly diagnosed patients including 11 with MGUS, 132 with MM and nine with PCL. The unsupervised analysis of the gene expression data profiled on high-density oligonucleotide microarrays identified two major groups: one including the majority of MGUS patients and normal controls, and the other all the PCL and most of the MM cases. Therefore, neither the MGUS, nor the PCL and MM samples could be identified as distinct entities. A multi-class analysis revealed probe sets specifically distinguishing MGUS from PCL, with the MM cases showing their progressively modulated expression. The MGUS cases showed the up-regulation of immune response genes, whereas the PCL cases showed the positive modulation of primary metabolism, and cell cycle and apoptosis induction. The hierarchical clustering generated in the 132 MM database was mainly driven by groups reflecting the TC classification.

We also analysed the GEP data in the context of distinct genetic lesions involving chromosomal gains or losses. All of the del(13) cases showed 67 down-regulated genes involved in protein biosynthesis, ubiquitination or transcriptional regulation, most of which (44/67) mapped along the whole chromosome 13.[10] In terms of 1q gain, the differential expression of 61 genes mainly localised on chromosome 1q12-1q44 distinguished MM patients with or without 1q extra copies. Functional analysis of the identified genes revealed their involvement in energy production pathways, intracellular protein transport, and stress-induced endoplasmic reticulum responses.[11]

Finally, the differential expression of 225 genes mainly involved in protein biosynthesis, transcriptional machinery and oxidative phosphorylation distinguished H-MM from NH-MM. Most of the up-regulated genes in H-MM mapped to the chromosomes involved in hyperdiploidy, whereas a significant fraction of the genes in NH-MM mapped to 16q.[12] Overall, the GEP data suggested a widespread gene-dose effect as the imbalances in expression closely correlated with the genomic structural abnormalities.

These results prompted us to use novel high-throughput approaches, such as high-density single nucleotide polymorphism (SNP) arrays, in an attempt to define the allelic imbalances that may contribute to the genomic instability and bio-clinical heterogeneity of MM. Furthermore, the integration of data derived from genome-wide DNA microarray analysis with transcriptional profiles may identify differentially expressed genes related to underlying chromosomal alterations, as being candidate tumour genes. We therefore used integrated FISH, GEP and whole-genome DNA SNP analyses to study a panel of 23 human myeloma cell lines (HMCLs) and identified some novel genetic imbalances.[13] Subsequently, we used the same approach in a study of 45 MM primary tumours included in the GEP dataset and, by means of a self-developed computational model based on combined FISH and genome-wide profiling analyses, found that marked aneuploidy characterised a significant fraction of the patients. In particular, an unsupervised analysis of the 45 genome profiles showed the presence of at least five main clusters of patients with different characteristics: an altered number of odd chromosomes suggesting hyperdiploidy; 1q gain and chromosome 13 deletion; deletions involving chromosomes 1p, 8p, 13, 22 and 14; aneuploidy (mostly near tetraploidy); and limited alterations. Non-parametric analyses using both the genomic and gene expression data identified a large number of genes whose expression closely correlated with copy number variations, thus further suggesting a marked gene-dose effect associated with allelic imbalances.

These findings provide a focus for further studies aimed at identifying and characterising genes that are involved in the pathogenesis of myeloma. To this end, we have started studies aimed at integrating FISH, GEP and SNP data with those derived from global microRNA (miRNA) gene expression analysis on microarrays. miRNA are small non-coding sequences that are thought to play important roles in regulating the genes involved in controlling cell cycle, survival and differentiation programmes, and are frequently located in hot spots for chromosomal abnormalities. Altered patterns of miRNA expression have already been demonstrated in a number of solid and hematological tumours.[14,15] The integration of multiple high-throughput approaches should increase the reliability and significance of our investigations, and provide synergistic information allowing the discovery of new pathogenetic networks and therapeutic treatments for MM.

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NEW MECHANISMS OF LEUKEMOGENESIS AND PROGRESSION IN THE PH+ ALL

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The Ph chromosome, the result of a reciprocal translocation fusing the abelson (ABL) proto-oncogene from chromosome 9 with the breakpoint cluster region (BCR) sequences on chromosome 22, represents the most common cytogenetic abnormality detected in human leukemias. It is considered the hallmark of chronic myeloid leukemia (CML), but is detectable in 20-30% of adult acute lymphoblastic leukemia (ALL), with the incidence rising to more than 50% of patients aged 50 years or older1. The BCR-ABL–encoded isoforms p210 and p190 recruit and activate multiple pathways that transduce oncogenic signals, leading to increased survival, enhanced proliferation, impaired migration and adhesion, and arrested differentiation of hematopoietic progenitors. The presence of the BCR-ABL1 rearrangement worsens the prognosis of ALL and represents the most significant adverse prognostic marker that significantly influences disease outcome. Current approaches to risk classification based on well-established clinical parameters, aberrant expression of antigens on surface of blast cells by immunophenotype analysis, early detection of minimal residual disease persistence after therapy and genetic lesions and aberrant expression profile of the blast cells.

The outcome for adult with Ph+ ALL has improved dramatically with current therapy including use of Tyrosine Kinase Inhibitors (TKIs) such as imatinib, nilotinib or dasatinib2-7. The complete hematological remission is obtained in about 100-98% of the patients treated with TKIs alone3,8or in association with conventional chemotherapy8, but relapse is an expected event in the majority of patients. The best characterized mechanisms of Bcr-Abl reactivation are Abl-kinase domain mutations which target critical drug contact points or, more often, induce a conformation in which drug binding is reduced or precluded.

Role of kinase mutations in Bcr-Abl dependent mechanisms of TKI resistance

The mechanisms by which mutations may confer resistance to imatinib fall into two categories. Some amino acid substitution occurs at critical contact points for imatinib binding.9-11 The most characteristic mutation of this category is the change of threonine to the bulkier and more hydrophobic isoleucine at residue 315, in the 'heart' of the imatinib binding pocket. The T315I was the first mutation described and remains the most difficult to treat. Other mutants at contact points for imatinib are exemplified by F317L and F359V.9 The second category, that includes the majority of kinase domain mutations, confers resistance via an indirect mechanism – that is, inducing a conformation of the kinase that imatinib no longer binds.9-11 This group includes mutants either favouring the active conformation of the kinase (M351T; H396R/P), or decreasing the flexibility of the P-loop (G250E, Q252H, Y253F/H, and E255K/V) so that the conformational changes required for imatinib binding cannot be adopted. Being expression of a generalized genetic instability, not all mutations that arise in the kinase domain may necessarily increase the Darwinian 'fitness' of the Ph+ clone. So, while some mutations do indeed drive resistance ('driver' mutations), other may simply be neutral bystanders that co-segregate with other determinants of resistance ('passenger' mutations).

Genetic instability and IKZF1 deletion

Data from murine studies demonstrate that expression of BCR–ABL1 in haematopoietic stem cells can alone induce a CML-like myeloproliferative disease, but cooperating oncogenic lesions are required for the generation of a blastic leukaemia. Notably, a number of genetic lesions including additional cytogenetic aberrations and mutations in tumour suppressor genes have been described in CML cases progressing to blast crisis. However, the specific lesions responsible for the generation of BCR–ABL1 ALL and blastic transformation of CML remain incompletely understood. A recent study by Mullighan et al. using high-resolution single nucleotide polymorphism (SNP) arrays12, has taken an important next step in the characterization of these genetic alterations and in so doing they highlight the power of high-resolution genome wide assessment for genetic alterations in cancer cells. This study assessed leukemia cell DNA obtained from 21 paediatric and 22 adult BCR–ABL1 ALL cases. Global assessment for copy number alterations demonstrated a mean of 8.79 somatic copy number alterations per BCR–ABL1 ALL case (range 1–26), with 1.44 gains (range 0–13) and 7.33 losses (range 0–25). No significant differences were noted in the frequency of copy number alterations between paediatric and adult BCR–ABL1 ALL cases. The most frequent somatic copy number alteration was deletion of IKZF1, which encodes the transcription factor Ikaros. IKZF1 was deleted in 36 (83.7%) of 43 BCR–ABL1 ALL cases, including 76.2% of paediatric and 90.9% of adult BCR–ABL1 ALL cases. Ikaros is the prototypical member of a Kruppel-like zinc finger transcription factor subfamily that is required for normal hemopoietic differentiation and proliferation, particularly in the lymphoid lineages. We recently showed13 that the expression of non-DNA binding Ikaros isoforms correlated with the BCR-ABL1 transcript levels in patients treated TKIs and their in vitro expression TKI sensitive cells increased proliferation and inhibited apoptosis, suggesting a pathogenic role in determining leukemia and/or resistance. Furthermore, we confirmed in 97 Ph+ ALL patients by SNP array and genomic PCR that the IKZF1 deletion is the most frequent somatic acquired alteration (Iacobucci et al. SIES 2008). The IKZF1 deletions were predominantly mono-allelic (64% vs 36%) and were limited to the gene in all cases, identifying IKZF1 as the genetic target. Two frequent deletions occurred in BCR-ABL1 positive: type A (38%) removing the exons 4-7 and type B (19%) generating a small isoform with only exons 1 and 8.

Other recurrent genetic alterations identified by Mullighan et al. and our group were deletions of PAX5 and of the CDKN2A/B locus. PAX5 encodes transcription factors required for normal lymphoid development. CDKN2A/B locus encodes three tumor suppressor genes that are widely inactivated in many human cancer.

Conclusions

Recent development of high-resolution SNP arrays has allowed detailed assessment of the genomes of Ph+ ALL cells and has demonstrated that that deletion of IKZF1 resulting in either haploinsuffciency and in the expression of dominant negative isoforms is an important event in the development of BCR-ABL1 B-progenitor ALL.

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ADVANCES IN IRON METABOLISM

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The identification of the liver peptide hepcidin has opened a new era in our understanding of iron metabolism. Studies of the iron deregulation in genetic hemochromatosis as well as characterization of cellular and animal models have led to the identification of the hepcidin pathway as the major pathway that regulates systemic iron homeostasis (for review see1). Hepcidin binds the iron exporter ferroportin and induces its internalization and degradation, in this way limiting the amount of iron released to the plasma from absorptive enterocytes and iron-recycling macrophages.[2]

Understanding hepcidin regulation is the present challenge. All hemochromatosis proteins are hepcidin modulators, the most important being hemojuvelin, since its inactivation produces the same juvenile hemochromatosis phenotype[3] of hepcidin inactivation.[4] Hemojuvelin is a coreceptor for Bone Morphogenetic Proteins (BMP),[5] linking cytokines and their signal transduction pathway through SMAD to the hepcidin pathway. A further link is shown by the SMAD4 liver conditional knock out mouse, which in unable to activate hepcidin and shows iron overload with features of genetic hemochromatosis.[6] Hemojuvelin is a complex protein belonging to the Repulsive Guidance Molecule (RGM) family: however at variance with RGMa and RGMb which are expressed in the nervous system, hemojuvelin is highly expressed in the skeletal muscle, heart and liver. It exists as a membrane-associated protein (m-HJV), the BMP coreceptor, which amplifies the hepcidin stimulation, and as a soluble form (s-HJV), which, as a decoy, inhibits hepcidin activation, binding and sequestering the BMP ligand. In the present model BMP 2/4/6, whose transcripts are present in the liver, are able to activate the hepcidin promoter in a luciferase assay in hepatoma cells.[5] BMP2 is also able to increase hepcidin production when injected *in vivo* in mice.

HFE, which can bind both transferrin receptor 1 (TFR1) and TFR2 in different iron status (mirrored by different transferrin saturation), is the likely sensor of plasma iron. Recently it has been shown that if unable to bind TFR1 HFE causes increased hepcidin through binding to TFR2.[7] TFR2, which is stabilized by diferric transferrin, is presumed to contribute to the sensing of circulating iron.

Hepcidin deficiency is the central pathogenic mechanism of genetic hemochromatosis,[8] but contributes also to the secondary iron overload observed in the iron-loading anemias, including the thalassemic syndromes.[9] These conditions, especially the transfusion-independent thalassemia intermedia, are characterized by increased iron absorption and ineffective erythropoiesis. In addition since hepcidin belongs to the family of antimicrobial peptides, its production is upregulated by inflammatory cytokines, especially by IL-6 and IL-1. Increased hepcidin levels explain the iron abnormalities in anemia of chronic disorders, which are characterized by iron sequestration in macrophages and iron-restricted erythropoiesis.

The molecular mechanisms underlying hepcidin downregulation are complex and not completely understood. Inhibition of hepcidin occurs in anemia, hypoxia/iron deficiency and erythropoiesis expansion. Hypoxia inducible factor 1 (HIF-1α) has been demonstrated to inhibit hepcidin in hypoxia.[10] s-HJV inhibits hepcidin *in vitro* competing with m-HJV.[11] *In vivo* injection of high dose of s-HJV three times/week for three weeks caused hemochromatosis in mice, indicating that s-HJV can regulate iron absorption.[12] We and others have shown that hemojuvelin has a furin consensus cleavage sequence RNRR at position 332-335,[13,14] and that the cleavage occurs in iron deficiency/hypoxia.

Growth Differentiation Factor 15 (GDF15), a member of the TGF-β superfamily, at high concentration partially inhibits hepcidin in hepatocyte primary culture *in vitro* and, since it is released in large amounts by the erytroblasts in patient sera, has been proposed to mediate hepcidin suppression in thalassemic patients.[15]

The increased knowledge in the field has allowed the identification of novel inherited disorders, as Divalent metal transporter 1 (DMT1) and Transmembrane serine protease 6 (TMPRSS6) defects, both characterized by microcytic anemia unresponsive to iron treatment. Understanding the molecular interrelationship of proteins that regulate iron homeostasis has the potential to identify novel therapeutic targets for patients affected by iron disorders.

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Table 1. Proteins of iron metabolism and their related disorders

Protein	*Function*	*Related disease*
HFE	Iron sensor (?)	HH (type 1)
Hemojuvelin	Hepcidin activator	JH (type 2A)
Hepcidi	Inhibitor of iron absorption	JH (type 2B)
TFR2	Iron sensor (?)	HH (type 3)
Ferroportin	Cell iron exporter	HH (type 4)
DMT1	Cell iron importer	Anemia and iron overload
Transferrin	Plasma iron transporter	Anemia and iron overload
TMPRSS6	Hepcidin inhibitor	Iron refractory anemia

HH: hereditary hemochromatosis; JH: juvenile hemochromatosis

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HISTONE DEACETYLASE INHIBITORS FOR CANCER THERAPY

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Introduction. Cancer has traditionally been considered a disease of genetic defects such as gene mutations and deletions, as well as chromosomal abnormalities, that result in the loss of function of tumor-suppressor genes and/or gain of function or hyperactivation of oncogenes.[1,2] However, there is growing evidence that gene expression modulated by epigenetic changes is also crucial to the onset and progression of cancer.[3] Remodeling of chromatin between relatively *open* and *closed* forms has a key role in epigenetic regulation of gene expression. Histone

deacetylases (HDACs) are enzymes involved in the remodeling of chromatin, thus playing a key role in the epigenetic regulation of gene expression. So far, 18 human HDACs have been identified and classified into four groups based on their homology to yeast proteins.

Cancer, HDCAs and HDAC Inhibitors (HDACi). Aberrant recruitment of HDACs to promoters through their physical association with oncogenic DNA-binding fusion proteins that result from chromosomal translocations, or overexpression of repressive transcription factors that physically interact with HDACs have been demonstrated in different types of leukemias and lymphomas. For example, the oncogenic PML-RARα, PLZF-RARα and AML1-ETO fusion proteins induce acute promyelocytic leukemia (APL) and acute myeloid leukemia (AML) by recruiting HDAC-containing repressor complexes to constitutively repress expression of specific target genes.[4] The transcription factor BCL6 which is overexpressed in nearly 40% of diffuse large B-cell lymphomas (DLBCLs) recruits HDAC2 to repress growth-regulatory target genes. BCL6 is itself hypo-acetylated by HDACs and treatment of DLBCLs with HDACi results in hyperacetylation of BCL6, release of HDAC2, reactivation of repressed target genes and tumor cell apoptosis.[5] Overexpression of individual HDACs has also been reported in prostate, gastric, colon, gastric, and breast carcinomas. Therefore, the identification of cancer-related epigenetic changes provide a strong rationale for the use of HDACi to reverse aberrant epigenetic states associated with cancer.[6] In fact, relieving transcriptional repression mediated by oncogenic transcriptional repressors might be sufficient to induce apoptosis, cell cycle inhibition and differentiation. Indeed, the scenario is more complex and indicates that the therapeutic effects of HDACi are not driven by reversing aberrant transcription, but rather through the differential effects on the malignant epigenome that result in the tumor-specific induction of pro-apoptotic genes.

Biological activities of HDACi. A large number of structurally diverse HDACi have been purified from natural sources or synthetically developed. HDACi can be divided into six classes based on their chemical structure, and these agents inhibit the enzymatic activity of HDACs with varying efficiency.[7] In preclinical studies, several classes of HDACi have been found to have potent anticancer activities, with remarkable tumor specificity. Evidences supporting tumor-selectivity emerge from the results of clinical trials and preclinical animal experiments demonstrating that HDACi can have potent anticancer activities at concentrations that are minimally toxic to the host. In vitro and in vivo studies have shown that HDACi kill tumor cells through apoptosis and cell-cycle arrest at the G1/S.[8] Additionally, HDACi have anti-angiogenic, anti-invasive and immunomodulatory activities that can contribute to the inhibition of tumor development and progression.[8] The anti-angiogenic properties of HDACi have been associated with decreased expression of proangiogenic genes.[9] HDACi also downregulate expression of the chemokine receptor 4 (CXCR4), which is important for the homing of bone-marrow progenitor and circulating endothelial cells to sites of angiogenesis.[10]

HDACi in Clinical Trials. At least 12 different HDACi used as monotherapy or in combination are currently being evaluated in more than 100 phase I-III clinical trials in hematologic malignancies [myelodysplastic syndromes (MDS), leukemias, myeloproliferative disorders, cutaneous T-cell lymphoma (CTCL), DLBCL, follicular lymphoma, Hodgkin lymphoma (HL), and multiple myeloma(MM)] and solid tumors (lung, breast, pancreas, renal, and bladder cancers, melanoma, glioblastoma).[11-14]

Early clinical studies showed substantial activity in relapsed and refractory CTCL, resulting in vorinostat being approved for this indication by the FDA. Phase II studies using vorinostat in relapsed and refractory patients demonstrated response rates (RR) of 30% and complete remission (CR) rates of 1-6%. Median response duration was 5-6 months, with a similar time to progression. Depsipeptide is currently in phase II clinical trials including a pivotal trial in CTCL and peripheral T-cell lymphomas. This drug has shown a RR of 32-39% in CTCL and a RR of 28% in a variety of subtypes of peripheral T-cell lymphomas. There is evidence for responses to HDACi in other hematological disorders, such as DLBCL and HL. A trial of vorinostat reported a 21% RR in 41 patients with relapsed or refractory leukemias or MDS. Depsipeptide has shown moderate activity in chronic lymphocytic leukemia (CLL) and AML, and stabilization of disease in MM. ITF2357 is currently in phase I-II clinical trials for refractory MM, HL, and mutated JAK2 myeloproliferative disorders. Throughout these trials toxicities have been minimal. There are clear potent anticancer effects with striking clinical responses observed in some patients, although single-agent HDACi generally shows moderate responses. The biological effective doses of HDACi as well as the need for maintenance treatment in indolent diseases remain unclear.

Given their pleiotropic anticancer activities and their minimal toxicity to normal cells, HDACi are currently being evaluated in combination with other agents. HDACi are synergistic or additive with different anticancer agents, including radiation therapy, chemotherapy, differentiation agents, epigenetic therapy, and new targeted agents. Combination studies with all-trans retinoic acid or the DNA-demethylating agent 5-azacytidine are under investigation in hematological and non-hematological cancers. Combinations of HDACi and conventional chemotherapeutic drugs including gemcitabine or paclitaxel are under investigation phase II and III trials in non-small-cell lung cancer and pancreatic cancer. The capacity of HDACi to induce the expression of a large number of death receptors and ligands, coupled with a lower apoptotic threshold in HDACi-treated cells, provides a strong molecular basis for combination studies using HDACi and the death receptor ligand TRAIL. A strong rationale also exists for exploring the activity of HDACi in combination with kinase inhibitors and proteasome inhibitors.

The multiple protein targets of HDACs and HDACi as well as preclinical evidences of synergy and additive activity with many other anticancer agents suggest that therapeutic strategies using HDACi in combination with other anticancer agents may be most promising.

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STEM CELL TRANSPLANTATION IN AMYOTROPHIC LATERAL SCLEROSIS PATIENTS

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Stem-cell-based therapies represent the hope to restore and preserve function in the brain and spinal cord of patients with neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS).[1-3] ALS is a devastat-

ing incurable neurodegenerative disease which targets motorneurons (MNs). It manifests as a linear decline in muscular function eventually leading to paralysis, speech deficits and dysphagia. Within 2 to 5 years of clinical onset death, due to respiratory failure, occurs.[4] Human Mesenchymal Stem Cells (hMSCs) are multipotent stem cells which are very attractive in view of a possible cell therapy approach in ALS because of their great plasticity and their immunological proprieties.[5-8] We isolated MSCs from healthy donors' bone marrow and performed experimental pre-clinical studies to: 1) investigate their possible neural fate *in vitro*, from morphological, biochemical, gene expression and physiological points of view and 2) test the effects of injecting MSCs into the spinal cord on the progression of ALS in using transgenic $SOD1^{G93A}$ mice. *In vitro* studies: MSCs were isolated from healthy donors using Percoll gradient and analyzed for cellular growth, viability and immunophenotype analysis for CD45 and CD14, CD90, CD29, CD44, CD105, CD166 and CD106. In order to investigate their possible neural fate, we tested hMSCs in 3 previously reported experimental conditions made of α-MEM/1 mM β-mercaptoethanol (βME), α-MEM/retinoic acid 10 μM or α-MEM/2% dimethylsulfoxide + 200 μM β hydroxyanisole respectively and in a new experimental condition in which different growth factors were added to a Neural Progenitor Maintenance Medium (NPMM). We analyzed the morphological changes, the protein and gene expression of neural markers such as Nestin, Neuron Specific Enolase (NSE), Neuronal Nuclei (NeuN), Microtubule Associated Protein (MAP2), Glial Fibrillary Acidic Protein (GFAP) and the electrophysiological proprieties. In βME, the cells became immunoreactive for NeuN, NSE and GFAP. In experimental conditions 2 and 3, MSCs were NeuN and NSE-positive while in NPMM they were positive for GFAP and NSE. In all experiments, MSCs expressed mRNA for MAP2, NSE, and NF-M but not for GFAP either before or after neural induction. In the electrophysiological study, NPMM-differentiated hMSCs expressed two delayed rectifier K^+ currents related to two ether-à-go-go K^+ channels (eag1, eag2) which are fundamental for setting the negative resting potentials required for neuronal survival and basal cell activity and one Ca^{2+}-dependent K^+ current. The three K^+ channels were absent in undifferentiated hMSCs. Thus, in our culture conditions, hMSCs acquired new morphological characteristics, neural markers and electrophysiological properties, some of which are suggestive of neural differentiation.[9]

Moreover, we observed that MSCs isolated from bone marrow of ALS patients maintain all their peculiar MSC characteristics and their extensive in vitro expansion does not involve any functional modification including chromosomal alterations or cellular senescence. ALS patients' MSCs acquire, under specific conditions, new morphological characteristics and neural markers, which are suggestive of neural differentiation as in healthy donors.[10]

For *in vivo* studies we transplanted hMSCs labelled with bismenzimide, into the lumbar spinal cord of SOD1G93A mice, bearing a mutation in the SOD1 gene, an experimental model of ALS, before the onset of symptoms. The study included SOD1G93A mice transplanted with hMSCs (n=25) and sham-operated transgenic mice (n=9). We also considered the sex of the mice (n=16 males and n=18 females), since it has been reported that the progression has a different time course in the two sexes (11). At the age of 38 weeks the mice were sacrificed and tissue analysis were performed on lumbar spinal cord to evaluate: 1) the presence of human cells (bismenzimide positive cells) and of neural markers; 2) *the quantification of alpha motoneurons;* 3) the reactive astrogliosis and 4) microglial activation by Immunohistochemistry for microglial antigen (CD11b). Moreover, in order to analyse the effect of MSC transplantation on the onset and progression of motor symptoms in this disease model, we performed four clinical tests: i) scoring of motor deficits, ii) weighing and iii) performance on the Rotarod task, all of which are commonly used to evaluate $SOD1^{G93A}$ animals.[12] In addition, we investigated iv) the paw grip endurance (PaGE) test which has the dual advantage of measuring motor strength directly while requiring only minimal equipment. hMSCs were found in the spinal cord 10 weeks after transplantation, sometimes close to motoneurons. They were rarely labelled for GFAP or MAP2. In females, where the progression of ALS is slower than in males, astrogliosis and microglial activation were reduced and motoneuron counts with the optical fractionator did show significant decreases in cell loss in hMSC-injected mice compared to sham-operated ones. Motor tests (Rotarod, PaGE, neurological examination) showed a significant improvement in transplanted males. Therefore, hMSCs can provide immunomodulatory and trophic substances, and are very promising tools also as vectors into the damaged spinal cord of ALS patients.

The preclinical *in vitro* e *in vivo* studies on MSCs in ALS patients were encouraging and early clinical investigations in a pilot study indicated that transplantation of autologous MSCs into the spinal cord was feasible in ALS patients.[13-14] However, as these studies were limited to 9 patients, the safety of intraspinal transfer of MSCs for ALS remains uncertain, hence we performed another Phase I trial to assess the feasibility and toxicity of the procedure. The trial was approved and monitored by the Italian Institute of Health and by the Ethic Committees of all participating institutions. Autologous MSCs were isolated from bone marrow, *in vitro* expanded and analyzed according to GMP conditions. Expanded MSCs were suspended in the autologous cerebrospinal fluid and directly transplanted into the spinal cord with a surgical procedure. Patients were strictly monitored before and after transplantation by clinical, psychological, neuroradiological and neurophysiological assessments. Ten ALS patients were enrolled. No telomere shortening or chromosomal alteration was noted in expanded MSCs in any patients. There was no immediate or delayed transplant related toxicity. Clinical, laboratory, and radiographic evaluations of the patients showed no serious transplant related adverse events. This study demonstrates that transplantation of MSCs into the spinal cord of ALS patients is safe and that MSCs might have a clinical use in ALS for cell based therapy.[15]

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CELLULAR THERAPY FOR AUTOIMMUNE DISEASES

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Autoimmune Diseases (ADs) are an heterogeneous group of diseases for genetics, clinical presentation and response to treatment which is usually long-term immunosuppression. In most of ADs there is a subset of patients either progressing on standard therapy or requiring a dose escalation of immunosuppression, thus resulting in a detrimental effect on quality of life, disability and overall survival. Autologous Haematopoietic Stem Cells Transplantation (HSCT) has been increasingly employed in the last ten years for the treatment of the latter group

of patients, showing the capability of inducing a clinical response, often treatment-free, in most of the patients. Durability of response is widely variable according to diagnosis, treatment intensity and disease phase at baseline. Transplant Related mortality (TRM) shows a trend to decrease, mostly due to both a better patients selection and the use of less intensive regimens. About 1500 patients have been reported to both EBMT and CIBMTR registries. Multiple Sclerosis is the most frequent diagnosis, with an overall Progression Free Survival (PFS) of 50% at 5 years and an overall TRM of 3.3%. Systemic Sclerosis (SSc) is the second reported diagnosis in the EBMT Registry, showing a similar outcome. Systemic Lupus Erythematosus, Crohn's Disease and autoimmune cytopenias have been also reported in the last 10 years with encouraging results. Type I Diabetes was recently reported in a pilot phase 2 trial. Prospective, comparative trials are currently ongoing both in Europe and North America, aimed to provide an evidence of the efficacy of HSCT over conventional treatments. Due to the expected high TRM, Allogeneic HSCT is to be considered only in very selected patients. In the last few years an increasing interest has been raised about the use of Mesenchymal Stromal Cells (MSC) for the treatments of severe ADs, after preliminary evidence of their activity in animal models of ADs and in the treatment of severe GVHD.

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ADVANCES IN ANTIFUNGAL THERAPY

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Fungi, notably *Aspergillus* and *Candida* species, are held liable for two-thirds of all superinfections in all patients who are treated for a malignant hematological disease.[1-3]

Under normal circumstances the intact epithelial surface and the mucociliary barrier does prohibit invasion and/or aspiration of fungal cells and spores but high doses of cytoreductive agents and intensive irradiation as part of the conditioning regimen do not only cause neutropenia, they also inflict serious damage to the mucosal barrier. This mutilation, which is not limited to the visible part of the mucosa, is likely to interfere with all tasks of the gut, including its protective function.[4,5] Under these circumstances colonizing micro-organisms can get easy access to the body and when disruption of the integument combines with neutropenia the patient is at double jeopardy.

Timely recognition of systemic fungal disease is a major prognostic factor. In spite of a muted inflammatory response, clinical signs and symptoms remain important diagnostic tools next to culturing, imaging techniques and, more recently, serological tests. Clinical findings are not only important in the selection the appropriate diagnostic tools, the clinical pattern also offers a clue to type of infection (see Table 1).

Table 1. Signs and symptoms suggestive of invasive fungal disease

All fungal infections
Persisting or new fever in patients known to be colonized by a fungus
Yeasts
-retrosternal pain, upper abdominal discomfort
-increasing alkaline phosphatise with persisting undulating fever
-multiple small hepatosplenic lesions ("bull's eyes") on imaging
-chorioretinal lesions upon return granulocytes
-fever in combination with unexplained rash and muscular tenderness
-macronodular cutaneous lesions, sometimes partly necrotic
-unexplained pain in spine or other bones, arthralgia
Molds
-dry cough with persisting fever
-chest pain related to respiration movements
-single or multiple pulmonary infiltrates, particularly 'halo' signs
-air crescent sign upon return granulocytes
-elevated antigen levels, particularly increasing titers
-facial pain with abnormality on nasal sinus X-ray
Pneumocystis jiroveci
- progressive hypoxemia with dry cough

Yeast infections, *Candida* being the most important representative, usually enter the body from the gastrointestinal tract or a damaged skin to cause a local infection that, in turn, offers access to the bloodstream. Fungemia is usually accompanied by signs of sepsis but the symptoms might be more subtle due to concurrent corticosteroids. If candidemia is not adequately treated all major organs might become affected although symptoms of this dissemination often only appear after bone marrow repopulation. Patients with a single positive blood culture, including those obtained via an intravenous catheter, should be treated immediately. The lungs and, to a lesser extend, the paranasal sinuses are the key organs in the pathophysiology of infections by molds. In most cases, fever refractory to antibacterial therapy or a localized pulmonary infiltrate displayed on high resolution CTscan of the chest constitute the first indication of invasive aspergillosis. If left untreated pulmonary aspergillosis will disseminate hematogenously to all organs. The clinical picture of zygomycosis (mucormycosis), fusariosis and alternariosis and other mold infections is not fundamentally different from that seen aspergillosis.[6] Although histopathology and culture remain the cornerstones of diagnosing an invasive fungal infection, serologic techniques are clearly gaining territory[7,8] ELISA techniques to detect galactomannan antigen in the serum proved useful in the management of invasive aspergillosis. Particularly the negative (optical density level <0.5) predictive value of the test is very high. Systematic screening of patients for the presence of galactomannan in the patient's serum may assist in the decision to start or stop antifungal therapy.[7] Unfortunately, there is no commercially available standardized probe for this very sensitive PCR technique.[11]

Facing the possibility of invasive fungal disease the physician has to address two questions: "When" to treat and "What" to treat with. Both prophylactic and empirical strategies that were designed to enable a timely intervention gained great popularity. The arrival of voriconazole and posaconazole has rekindled the interest in anti-*Aspergillus* prophylaxis. The potential of these drugs in the prophylaxis of invasive aspergillosis clinical studies has been explored and their outcome led to the conclusion that the prophylactic use of these azoles should be considered in high risk situations.[9]

Empirical therapy is instigated when the existence of an occult invasive infection cannot be reliably excluded. The classic situation involves fever refractory to broad-spectrum antibiotics for 3 to 5 days. Vigorous pursuit of an empirical strategy is inevitably linked with unnecessary exposure to expensive and potentially toxic antifungal compounds. An optimal diagnostic work-up makes it possible to exclude the presence of invasive fungal disease with a high grade of certitude. Hence, employing a consistent scheme for screening of febrile patients, it appears safe to refrain from antifungal therapy in patients without any further evidence of disease (Figure 1).[10] Centers with a high incidence of invasive fungal infections and no easy access to diagnostic facilities should put their stakes on a liberal prophylactic and empirical use of systemically active antifungal compounds, whereas for well equipped centers with a

low incidence a pre-emptive approach would suffice.

The first step in the management of invasive fungal infections should be a careful consideration of the clinical situation and assessment of the risk factors that might have contributed to the emergence of fungal disease. Once it has been decided that an antifungal drug is required, a therapeutic dose should be given. It is a misconception that the dose of an antifungal should vary with the indication. Tissue and serum levels should be within the therapeutic range, independent of indication or the number of micro-organisms that have to be eradicated.

Although most antifungals offer reliable activity against *Candida* spp, echinocandins appear to be the drugs of choice for initial treatment of an established or presumed Candida infection. The selection of the proper antifungal compound is often trivial. In the majority of cases the identity of the causative fungal pathogen is unknown, which urges to s cover of yeasts and locally prevalent molds if the indication for antifungal therapy is based on persistent fever only. Voriconazole and liposomal amphotericin B are generally considered to be the most potent drugs against aspergilllosis.[11] Caspofungin offers a safe alternative but its anti-*Aspergillus* activity is still disputed. Posaconazole is only available as an oral formulation and was never tested in critically ill patients or as first line therapy. Combining antifungal compounds remains an experimental treatment option.

The decision to start antifungals may appear complex but is not as difficult as the decision to discontinue. In the patient who appears ill or is at high risk, maintenance of antifungal therapy throughout the neutropenic episode is recommended. Conversely, when neutropenic fever subsides and the patient is free of symptoms, antifungals may be discontinued, particularly when the criterion for commencing antifungal therapy was simply fever unresponsive to antibiotics. If a systemic fungal infection has been identified, it is crucial to see a response before one ponders cessation of antifungal therapy.

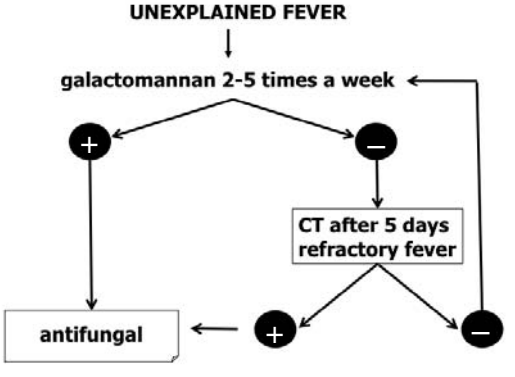

Figure 1. Diagnostics guided antifungal therapy for patients with unexplained fever.

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BEST ABSTRACTS

BEST-01

CHRONIC MYELOID LEUKEMIA WITH GENOMIC DELETIONS ON OTHER CHROMOSOMES INVOLVED IN VARIANT T(9;22)

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The t(9;22)(q34;q11), generating the Philadelphia (Ph) chromosome, is found in more than 90% of patients with chronic myeloid leukemia (CML). Large deletions adjacent to the translocation junctions were identified on the derivative 9 chromosome in 15-18% of CML cases with a classic t(9;22) and in 40–60% of patients bearing variant Ph translocations. To date, the biological significance of this genomic loss in the pathogenesis of CML is unknown. The most plausible hypothesis is that the loss of a tumor suppressor gene may confer a proliferative advantage to the Philadelphia-positive clone. We report a molecular cytogenetic study performed on 404 CML patients showing the occurrence of variant t(9;22) translocation in 36 cases (9%) of the analyzed series. These patients were characterized in details by Fluorescence In Situ Hybridization (FISH) analysis with P1 Artificial Chromosome (PAC) or Bacterial Artificial Chromosome (BAC) clones specific for ABL1 and BCR genes, as previously reported. A set of BAC/PAC probes belonging to chromosomes 9, 22 and to the third chromosome involved in variant rearrangements was selected querying the University of California Santa Cruz (UCSC) database. Chromosome 9 and/or 22 sequences loss was identified in 54 (15%) out of 368 CML cases bearing a classic t(9;22)(q34;q11) and in 14 (39%) out of 36 CML cases with variant t(9;22) rearrangements. Among these 14 patients, 9 (64%) showed microdeletions of sequences belonging to the third partner chromosome, revealing an high incidence of this kind of microdeletions and an association between the loss of genomic sequences of other chromosomes involved in variant t(9;22) and the deletions on der(9). Three-way variant translocations involving different partner chromosomes were detected in all these 9 cases. The deletions were identified in each analyzed metaphase, indicating that the rearrangement and the sequences loss occurred presumably at the same time. The size of the deletions at the third chromosome appears to be heterogeneous, ranging from 0.5 to 5.3 Mb. Moreover, the deleted sequences on the third derivative chromosomes included tumor suppressor genes (TSGs), genes involved in signal transduction or in modulation of cell proliferation, and microRNAs (miRNAs). The observation that deletions on the third chromosome involved in complex t(9;22) are associated with the loss of TSGs and/or miRNAs suggests their possible involvement in the CML pathogenesis, mediated by a haplo-insufficiency mechanism.

BEST-02

RELATION BETWEEN V617F JAK-2 GENE MUTATION AND THE PLATELET VON WILLEBRAND FACTOR, ADAMTS-13, AND NEUTROPHIL ACTIVATION MARKERS IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA

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Thrombohemorrhagic episodes are a common complication of essential thrombocythemia (ET). Recently, the JAK2V617F acquired mutation has been described in about half of patients with ET, and clinical data suggest an association between this mutation and higher rate of thrombosis. Von Willebrand factor (vWF) is a multimeric protein stored in endothelial cells and platelets. Upon cell activation, vWF ultra large multimers are released into the blood, where they are cleaved in smaller units by the metalloprotease ADAMTS-13. Because in ET patients, platelets and neutrophils circulate in an activated status, we evaluated in a group of 69 consecutive patients (52% JAK2V617F carriers) the followings: 1) platelet and plasma vWF content; 2) plasma ADAMTS-13 activity levels; and 3) the distribution of these parameters between patients with and without JAK2V617F mutation. Total vWF antigen (vWF:Ag) and activity (vWF:Act) were determined in both plasma and isolated washed platelets. ADAMTS-13 activity was assayed in plasma according to Gerritsen's method. CD11b was measured as an activation cell marker on neutrophil membrane by flow cytometry. Significantly higher plasma levels of both vWF:Ag and vWF:Act were measured in ET patients compared to controls ($p<0.05$), with no statistically significant differences found between JAK2V617F positive and negative patients. The vWFAct/Ag ratio was significantly ($p<0.01$) lower in ET patients *vs.* controls. A statistically significant inverse relation was found between platelet count and plasma vWF:Act. vWF evaluation in platelets showed a significant reduction of both vWF:Ag and vWF:Act in ET patients compared to controls ($p<0.01$), thus suggesting an increased release of vWF from platelets. In addition, the JAK2V617F positive patients had the lowest platelet vWF:Act values ($p<0.01$) and vWFAct/Ag ratio ($p<0.01$) compared to JAK2V617F negative patients and controls. An inverse correlation was found between CD11b levels and platelet vWF:Act ($p<0.01$). Plasma ADAMTS-13 activity was slightly reduced in ET patients ($p<0.01$), but no relation was found with plasma and platelet vWF. In conclusion our data demonstrate a decrease in platelet vWF:Ag and Act in ET patients, particularly in JAK2V617F carriers. The reduction correlates to neutrophil activation, suggesting a role of neutrophil in platelet degranulation. Increased vWF release and reduced ADAMTS-13 activity is a recognized mechanism of hypercoagulation.

BEST-03

CHARACTERIZATION OF A CELLULAR MODEL FOR THE STUDY OF MITOCHONDRIAL FERRITIN PHYSIOPATHOLOGICAL ROLE IN SIDEROBLASTIC ERYTHROPOIESIS

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Recently, in the erythroblasts of refractory anemia with ring sideroblasts (RARS), we identified a novel mitochondrial ferritin (MtFt) that may play an important role in regulating iron homeostasis and toxicity. MtFt may protect mitochondria from oxidative damage and increase cell resistance to the apoptotic signals. To clarify the possible role of MtFt in the ineffective erythropoiesis of RARS, we assessed its distribution in RARS erythroid progenitors and searched for possible correlations between MtFt expression and erythroid maturation, apoptosis, proliferation, clonogenic capacity. $CD34^+$ bone marrow cells from 24 patients with RARS, 16 patients with refractory anemia (RA) and 8 healthy donors were cultured for 14 days in a liquid medium according to a procedure which allows the expansion of high numbers of erythroid progenitors in the presence of Il-3, IL-6, stem cell factor and erythropoietin. At days 0, 4, 7, 11 and 14, cytospins were performed for MtFt, HFt and LFt immunocytochemical analysis, and samples of cultured cells were removed for other biological studies. There was a continuous increase of erythroid cells during the culture period: at day 14, 65% of the cells were Glycophorin A+. In RARS and RA Glycophorin A^+ cells showed CD71 down-regulation ($p=0.0002$). At day 0, $CD34^+$ cells of all samples were negative for MtFt. RARS erythroid progenitors showed an early expression of MtFt (day 4) and a continuous increase during the culture (5-24%, day 4-14). LFt levels were variable, while HFt expression was higher in RA ($p<0.001$). Cell growth was lower in RARS and RA than in normal controls ($p<0.0001$). Whereas in normal samples cycling cells increased step by step (69%, day 14), in pathological samples they remained stable (26%, day 14). RARS and RA progenitors showed a tendentially higher apoptotic rate and an inverse correlation between apoptosis levels and BFU-E number. In RARS, apoptotic levels were tendentially higher in $MtFt^+$ cells than in MtFt- cells ($p=0.06$), whereas in RA a positive correlation between apoptotic rate and HFt expression was observed ($p=0.001$). In conclusion, we have demonstrated an abnormal iron-loaded phenotype in RARS and RA erythroid progenitors characterized by MtFt or HFt overexpression and transferrin receptor down-regulation. The association of this phenotype with low proliferation and increased apoptosis suggests a close relationship between impaired iron metabolism and pathogenesis of myelodysplasia.

BEST-04

CD34-POSITIVE HEMATOPOIETIC PROGENITOR CELLS ARE INVOLVED IN ACUTE MYELOID LEUKEMIA WITH NPM1 GENE MUTATION

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Acute myeloid leukemia expressing mutated NPM1 gene and cytoplasmic nucleophosmin (NPMc+AML) [Falini *et al*., NEJM 352:254, 2005] is a new entity of WHO classification that shows distinctive biological and clinical features, including a unique molecular signature characterized by downregulation of CD34 and upregulation of most HOX genes [Falini *et al*., Blood 109:874, 2007]. Involvement of HOX genes in the maintenance of the stem-cell phenotype strongly suggest that AML with mutated NPM1 originates from a multipotent hematopoietic progenitor (HSC). This view is also supported by immunohistological findings showing that AML with mutated NPM1 embraces a wide spectrum of FAB morphologies (with the exception of M3, M4eo and M7), and frequently displays multilineage involvement [Pasqualucci *et al.*, Blood 108:4146, 2006]. On the other hand, the frequent negativity of NPMc$^+$ AML for the HSC-associated antigen CD34 raises the question of whether the mutation event occurs in a CD34-negative HSC (these cells have been identified in mice) or whether a pool of CD34-positive NPM1-mutated leukemic cells does exist. To address this issue, we purified CD34$^+$ cells from NPMc$^+$ AML patients and detected NPM1 mutant protein in the sorted population by Western blot with anti-NPM mutant specific antibodies. We investigated 6 NPMc+ AML patients presenting at diagnosis with 0.12%, 0.14%, 0.38%, 5%, 22%, and 28% of CD34$^+$ cells in the peripheral blood. In all cases, CD34$^+$ fractions (purity >90%) harbored NPM1 mutant protein, indicating they belong to the leukemic clone (Figure 1). The percentage of most undifferentiated CD34$^+$/CD38$^-$ cells in the CD34$^+$ fractions ranged from 5 to 97%. Notably, in at least one case, all CD34$^+$ NPM1-mutated leukemic cells were CD38-negative, suggesting they belong to the pool of leukemic stem cells. Inoculation of CD34$^+$ NPM1-mutated AML cells into sublethally irradiated NOD/SCID mice resulted into leukemia engrafment in various body sites, especially bone marrow, spleen, lung and liver. Preliminary results show cells differentiated and reacquired the same leukemic phenotype as the original patient's. These findings suggest the CD34$^+$ fraction contains the SCID-leukemia initiating cells (SL-IC) and point to CD34$^+$/CD38$^-$ HSC as the cell of origin of AML with mutated NPM1. Whether this cell belongs to the compartment of HSC or Common Myeloid Progenitor (CMP) cells (upon re-programming) and how NPM1 mutation confers a selective myeloid differentiation advantage remains to be clarified.

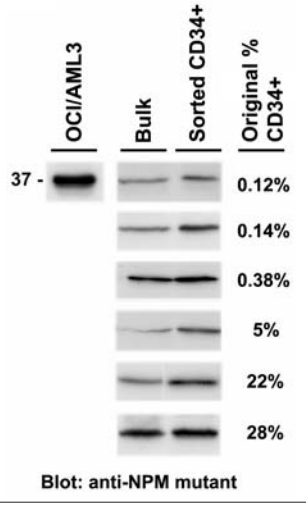

Figure 1.

BEST-05

FOXO3 TRANSCRIPTION FACTOR IS DELOCALIZED AND INACTIVATED IN ACUTE MYELOID LEUKEMIA PATIENTS

Panuzzo C, Arruga F, Messa F, Bracco E, Rotolo A, Nicoli P, Morotti A, Maffè C, Iacobucci I, Martinelli G, Baccarani M, Saglio G, Cilloni D

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The FoxO transcription factor promotes apoptosis and triggers cell cycle inhibition through multiple mechanisms regulation such as PI3K/Akt induced phosphorylation, resulting in nuclear exclusion and degradation. Moreover, several data demonstrated an abnormal activation of PI3K/Akt pathway in AML blast cells. The aim of this study was to clarify the role of FoxO3 in AML and to investigate alternative pathways eventually responsible for FoxO3 inactivation. Protein amount and localization were analyzed by Western blot and immunofluorescence and the DNA binding activity was measured by EMSA. 35 BM samples from AML patients at diagnosis and in 20 healthy donors were analyzed. Furthermore Spred1, known to be a FoxO3 target gene was quantified by RQ-PCR. We previously described the absence of Spred1 in AML patients and demonstrated that it promotes growth arrest and apoptosis in haematopoietic cells. Finally BM cells were incubated with a PI3K inhibitor LY294002 and the IKK inhibitor PS1145, alone and in combination. Moreover, the t(8;21) positive Kasumi cell line was transfected with pECE-FoxO3 to evaluate FoxO3 effects on cell growth and apoptosis. We found that, while FoxO3 in control cells is localized in both nucleus (mean value of intensity of 21.4±2) and cytoplasm (14.6±1.7), it is completely cytoplasmatic in AML cells (18.1p4,6 in cytoplasm *vs.* 8,2±4 in the nucleus) and enters the nucleus after chemotherapy. Moreover, FoxO3 DNA binding activity in AML patients is completely absent at diagnosis and restored after therapy. Also the mRNA of Spred1 is rather undetectable at diagnosis (2-ΔΔCt= 0,009±0,3) and shows normal levels during remission (2-ΔΔCt = 2±1,5) or after LY29400 incubation (2-ΔΔCt =0,8±0,3). In addition LY294002 and PS1145 treatment results in FoxO3 partial nuclear relocalization while their association induces a complete nuclear shuttle suggesting that both pathways could be implicated in FoxO3 inactivation. Finally, FoxO3 overexpression in transfected cells results in a block of proliferation rate (66% of inhibition compared to empty vector transfected cells). Taken together these data suggest that FoxO3 inactivation may be crucial for the leukemic progression and demonstrate that also IKK pathway contributes to this effect, providing the rationale for a therapeutic strategy based on the combination of selective inhibitors such as FLT3 or Akt inhibitors or standard chemotherapy and the IKK inhibitor.

BEST-06

CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED MICRO-RNAS IN HEMATOPOIETIC CELLS OF CHRONIC MYELOPROLIFERATIVE DISORDERS

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Additional molecular defects associated with MPD beyond JAK2 or MPL mutation, and possibly contributing to their variable phenotype, remain largely undefined. miRNAs emerged as regulators of gene expression, and we recently reported on miRNA profile in PMF granulocytes. The aim of this study was to identify abnormally expressed miRNAs in CD34$^+$ cells from patients with PMF, PV or ET. We studied 50 patients with PMF, 40 PV and 25 ET. Selected miRNAs were assayed using stem-looped primer technology and real-time PCR in granulocytes, CD34+ cells and erythroid colonies. Expression of miRNA precursors was analyzed with SYBR Green RT-PCR. PMF granulocytes could be differentiated from both PV and ET based on the expression of a set of four miRNAs, of which miR-31, -150, and -95 were downregulated and -190 was upregulated. We found increased expression of miR-182,-183 and -96 in granulocytes from all MPDs compared to controls or subjects with idiopathic or secondary erythrocytosis (n=15); these genes are included in the same cluster on chr7 and their level was correlated with JAK2V617F allelic burden. In-silico analysis indicated Bcl-2, whose expression levels were actually found increased in MPD cells, as potential target of this

miRNA cluster. Overexpression of miRNA16 was observed in CD34+ cells and in both EEC and Epo-dep BFU-E in patients with PV; the level measured in patient with post-PV myelofibrosis were significantly higher than in all other conditions. Additionally forced expression of miR-16 by transfection in erythroid progenitors induced overexpression GpA,CD36 compared to control. To address potential mechanisms for miR16 overexpression we performed FISH and sequencing analysis of microRNA precursors (miR16-1 and -16-2 that are located on chr.13 and chr.3, respectively), but no chromosomal nor sequencing abnormality was found. Also expression level of miR-15a and -15b, included in the same cluster with miR-16, were found unchanged compared to controls, suggesting a specific up-regulation of miR-16 due to a still uncharacterized mechanism. These data indicate that specific abnormalities in the expression of selected miRNAs can be detected in granulocytes and CD34$^+$ cells from MPD, that in some instances are correlated with JAK2V617F mutational status; in particular, hsa-miR-16 might represent a potential novel prognostic factor in PV patients since its expression level correlated with the progression of the disease towards myelofibrosis.

ORAL COMMUNICATIONS

Chronic Myeloid Leukemia and Myeloproliferative Disorders (I)

C001

EPHA3 KINASE IS CONSTITUTIVELY ACTIVATED IN CHRONIC MYELOID LEUKEMIA DURING ACCELERATED AND BLAST CRISIS AND CAN BE TARGETED BY DASATINIB OR BY MONOCLONAL ANTIBODIES

Arruga F, Carturan S Messa F, Chiarenza A, Catalano R, Defilippi I, Maffè C, Rotolo A, Iacobucci I, Martinelli G, Bracco E, Lackmann M, Saglio G, Cilloni D

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Eph receptors tyrosine kinase are involved in many key developmental processes. Ephs are overexpressed or mutated in solid tumours suggesting their possible role in oncogenesis. The mechanisms leading to CML progression are partially unknown. Therefore the aim of this study was to investigate the role of EphA3R in CML progression and to explore the possibility to target EphA3R with TK inhibitors and with a monoclonal antibody. The expression of EphA3R and ligands were analyzed by RQ-PCR in 52 samples from 36 CML patients and in 38 healthy controls. In 5 patients and 10 healthy subjects CD34$^+$ cells were enriched. Sequencing of TK domain was performed. Protein expression and localization were examined using WB and immunofluorescence analysis. The effects of EphA3R overexpression were studied by transfecting EphA3R in 293T e COS cells. Finally samples were incubated with Dasatinib 20nM for 6 hrs and evaluated for cell proliferation by incorporation of 3H timidine, for apoptosis by FACS and for colony growth. Normal BM, PB and CD34$^+$ cells and CP CML cells expressed very low levels of EphA3R. By contrast, during the advanced phases of disease (AP and BC) we found high transcript levels (mean of 2-Delta Delta Ct 43 and 67 respectively). Moreover, purified CD34$^+$ CML cells presented significantly higher levels as compared to the unfractioned sample (p=0,001). By contrast RT-PCR showed no increase of the expression of ligands EphA2,A3,A4,A5 and B2, all of them able to bind EphA3R. WB and immunoflourecence confirmed the presence of phosphorylated protein in EphA3 in AP and BC CML cells. Dasatinib incubation induced a significant inhibition of EphA3R phosphorylation. Moreover, Dasatinib induced significant apoptosis (mean value 32%±12), colony growth reduction (mean value of 34,2 *vs.* 76,5) and proliferation rate inhibition (48%±17) in EphA3$^+$ cells compared to normal controls and to EphA3 negative cells in which we were unable to observed any significant effect. Similar effects were observed after incubation with a specific antibody which acts by blocking the receptor. No kinase domain mutations were found in EphA3 overexpressing cells. In conclusion, EphA3 is abnormally expressed during CML progression. The inhibition of EphA3 phosphorylation induced by Dasatinib or by the antibody results in growth arrest and apoptosis of EphA3 overexpressing cells. Therefore, EphA3 may represent a potential candidate for a molecular therapy in advanced phase of the disease.

C002

EFFECT OF MTOR INHIBITOR RAD001 IN CELLS HARBORING THE JAK2V617F MUTATION

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Somatic mutations in JAK2 and MPL with constitutive activation of JAK/STAT pathway have been described in chronic myeloproliferative disorders (MPDs). On the contrary, little is known about phosphatidylinositol 3-kinase(PI3K)/Akt signalling, particularly of the mammalian target of rapamycin (mTOR). mTOR is often abnormally activated in cancer and represents an attractive target for anticancer therapy. We used RAD001, a specific mTOR inhibitor, to evaluate involvement of this pathway in MPDs. The IC50 of RAD001 in JAK2V617F+HEL cell line was 16 uM, similar to BCR-ABL+K562 cells (14 uM) but significantly higher than in leukemic KG1 cells (1.3 uM). On the contrary, the IC50

of murine Ba/F3 cells transfected with murine or human JAK2V617F (0.01 and 0.9 uM, respectively) was significantly lower than in Ba/F3/Parental cells or Ba/F3 transfected with wild-type JAK2 (4.3 uM). The EPO dependent Ba/F3 cells did not display any difference in IC50 when cultured with IL3, while in the presence of Epo the IC_{50} was 10-fold lower in JAK2V617F (0.03 uM) than in Ba/F3/P cells (0.6 uM) suggesting that EPO trigged mTOR-dependent pathway and increased sensitivity to the drug. We also used JAK inhibitor I (Calbiochem),a potent inhibitor of JAK1, JAK3, TYK2 with greater specificity for JAK2. HEL and KG1 presented lower IC_{50} (0.6 and 1.4 uM) than K562 (15 uM),while no difference was noted in Ba/F3 cells whether transfected or not with JAK2V617F. The effect of RAD001 in HEL cells was synergic with JAK inhibitor I, since the IC_{50} was reached with 3 uM RAD001 (1/5 of IC_{50}) and 0.4 uM JAK inhibitor I (IC_{50}=0.6 uM).HEL cells exposed to RAD001 IC50 presented increased G0/G1 phase and reduction of S phase, with higher number of apoptotic and necrotic cells. A reduction of phosphorylated mTOR and 4EBP1 (the main down-stream effector of mTOR) at 6h-12h after RAD001 was demonstrated, as well as early(1-3 hr) reduction of mRNA of mTOR target genes CCDN1 and GLUT-1.Clonogenic assays were performed using mononuclear or $CD34^+$ cells from control subjects or PV and PMF patients. While changes of CFU-G/GM in controls were minimal, there was a dose-dependent inhibition of both CFU-Mk and BFU-E in controls and MPD. These data suggest involvement of mTOR in MPD pathogenesis through enhanced EPO-dependent activation of JAK2V617F/STAT pathway and its possible relevance for treatment.

C003

HIGH-RESOLUTION MAPPING OF DELETIONS OF CHROMOSOME 9Q$^-$ IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA BY SNP-ARRAYS: WHICH GENES MATTER?

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Background. Extensive microscopic deletions 5' to ABL and/or 3' to BCR on the derivative chromosome 9 ($9q^+$) may be detected at diagnosis in some of patients (pts) with chronic myeloid leukemia (CML). $9q^+$ deletions ($del9q^+$) have been associated with adverse outcome in pts treated with chemotherapy. *Aims.* Last year we presented the results of a study conducted on 442 newly diagnosed CML pts enrolled in GIMEMA CML WP studies of imatinib . The rates of complete cytogenetic response and major molecular response were shown not to differ significantly between pts with (n=55) and without (n=387) $del9q^+$. It can be hypothesized that the size of the deleted region may be a confounding variable and that one or more critical genes lost in some cases and retained in others may be responsible for differences in pt outcome. In order to address this issue, we planned to use SNP-arrays to perform a high-resolution mapping of the deleted region in the 55 pts who carried $del9q^+$ (as assessed by FISH analysis). *Methods.* DNA could be extracted from bone marrow mononuclear cells archived at diagnosis in 42/55 pts. SNP-array-based karyotyping was perfomed using Affymetrix GeneChip Human Mapping 250K Nsp arrays. Copy number analyses were performed using Partek Genomic Suite software. *Results.* Results obtained in the subset of 13 pts analyzed so far show that the extension of genomic loss is variable in size. The deleted region spanned 600 kb to 2.3 Mb 5' to ABL and/or 150 kb to 7.8 Mb 3' to BCR. Detailed maps of genes involved were generated for each pt. The comparison of these maps highlighted clusters of genes that were lost in different subgroups of pts. On chromosome 9, they included the PP2A inhibitor SET; PPP2R4, encoding the regulatory subunit of PP2A; the oncogenic transcription factor PRDM12; TOR1A and TOR1B, two ATPases; on chromosome 22, they included MIF, a lymphokine involved in tumor cell motility; the splicing factors TFIP11 and SF3A1; the ADRBK2 receptor kinase; the mitotic spindle assembly checkpoint regulator MAD1; the regulator of chromatin SMARCB1; the Rgr oncogene, encoding a Ras activator. *Conclusions.* Molecular characterization of $del9q^+$ has so far been performed only by FISH. To the best of our knowledge, this is the first study allowing a high-resolution mapping of sequences involved in $del9q^+$. Clinical correlations with Sokal risk and response to imatinib treatment will be presented at the meeting. *Supported by PRIN, FIRB, AIL, AIRC, European LeukemiaNet.*

C004

REDUCED LEVELS OF SHP1 PROVIDES AN ADDITIVE SURVIVAL ADVANTAGE TO THE PH$^+$ CELLS OF CML PATIENTS AND MAY ACCOUNT FOR RESISTANCE TO IMA TREATMENT

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A part of chronic myeloid leukemia (CML) patients in chronic-phase demonstrates primary resistance to Ima or develop secondary resistance during treatment. We investigated the role of two non-receptor protein tyrosin phosphatases (Shp1 and Shp2) in the resistance to Ima. To this aim, we first used, as model system, a couple of Ima-sensitive and -resistant KCL22 cell lines (KCL22s and KCL22r), that are interesting since they do not show any of the known mechanisms of Ima resistance and, as the Ph+ stem cells, the original parental Kcl22s shows a high level of resistance to the Ima-induced apoptosis. We found a very low level of Shp1 (both mRNA and protein), a protein with a tumour suppressor activity, in the KCL22s respect to the KCL22r cells. Methylation-specific PCR analysis of Shp-1 promoter showed that the down-regulation of this gene is related to the methylation of its promoter. Indeed, 5-Azacytidine (5-AC) treatment, along with demethylation of the promoter region, re-induced expression of Shp1 in KCL22r, and this treatment also re-established the Ima sensitivity, i.e. Ima growth inhibition, in these cells. At molecular level, the restored Ima sensitivity was associated to a significant reduction of phosphorylation of both STAT3 and ERK1/2. To better understand the cellular processes in which Shp1 is involved, we carried out mass spectrometry to find Shp1 binding proteins, and found that Shp1 interacts in these cells with Shp2, a protein phosphatase well known as positive regulator of oncogenic pathways, including the Ras/MAPK pathway. Therefore, Shp1, through dephosphorylation, might modulate the activity of Shp2. Knocking-down of Shp2 in KCL22r, similarly to the up-regulation of Shp1, restored Ima sensitivity and on the opposite knocking-down of Shp1 in KCL22s cell line decreased its sensitivity to the drug, thus supporting the role of these protein in Ima sensitivity. Most importantly, the role of Shp1 appear to be important in the resistance to Ima treatment also *in vivo*. Indeed, we found that 5 CML patients who showed primary (n=3) or secondary resistance (n=2) to Ima, the levels of Shp1 mRNA were significantly reduced respect to CML patients matched for age and Sokal risk, who showed a clinical response to the treatment (mean level SHP1 3.98 for resistant patients versus 7.2 for those sensitive). Taken together, our results indicate that the balance between the levels of Shp1 and 2 may account for the Bcr-Abl independent activation of Ras/MAPK pathway and for the resistance to Ima.

C005

FOXO3 TRANSCRIPTION FACTOR IS DELOCALIZED AND INACTIVATED IN CML PATIENTS BY BCR-ABL ONCOGENIC SIGNALLING

Panuzzo C, Messa F, Arruga F, Bernardoni R, Rotolo A, Carturan S, Nicoli P, Morotti A, Marrè C, Iacobucci I, Martinelli G, Baccarani M, Saglio G, Cilloni D

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The FoxO family of transcription factors is regulated by PI3K/Akt induced phosphorylation resulting in nuclear exclusion and degradation. Nuclear FoxO transcribes proapoptotic molecules and cell cycle inhibitors. In CML cells the TK activity of Bcr-Abl leads to the abnormal activation of downstream effectors including PI3K/Akt. The aim of this study was to investigate the role of FoxO3 in Bcr-Abl induced apoptotic arrest and cell growth and the effect of imatinib (IM) treatment on FoxO3 activity. BM cells were collected from 30 CML patients and 20 healthy donors. The expression levels of FoxO3 were tested by RQ-PCR; protein amount and localization analyzed by Western blot and immunofluorescence and DNA binding activity measured by EMSA. FoxO3 was analyzed also in CML patient cells and Ph^+ cell lines after incubation with IM, LY294002 and PS1145. In addition K562 Ph^+ cells

was transfected with pECE-FoxO3 to clarify FoxO3 effects on cell growth and apoptosis. Finally we used our already set up model of Drosophila melanogaster (Dm) transgenic for human Bcr-Abl to study the pathways leading to FoxO3 inactivation. We found that, despite either FoxO3 mRNA levels or protein amount are similar in CML cells compared to controls, FoxO3 protein is equally distributed in the nucleus and cytoplasm in controls but it's completely cytoplasmatic in CML cells and it enters the nucleus during IM treatment. Additionally, FoxO3 DNA binding activity in CML patients is completely absent at diagnosis and reappears after IM, LY294002 or PS1145 treatment. Moreover FoxO3 overexpression in transfected cells results in a block of proliferation rate (49% of inhibition compared to empty vector transfected cells) while IM treatment in transfected cells results in a stronger effect with approx 75% of inhibition respect to the control. Finally, the progeny obtained from the crossbreeding of Bcr-Abl flies and flies transgenic for FoxO showed a rescue of FoxO phenotype demonstrating that FoxO inactivation is Bcr-Abl mediated. Taken together these observations suggest that FoxO3 is inactivated in CML cells and its delocalization is mainly dependant from Bcr-Abl activity. This results in increased proliferation and reduced apoptosis and it may be crucial for Bcr-Abl oncogenic pathway. The antiproliferative activity of IM may be mediated by FoxO3 relocalization.

C006

MOLECULAR PROFILE OF CD34+ STEM/PROGENITOR CELLS ACCORDING TO JAK2V617F MUTATION STATUS IN ESSENTIAL THROMBOCYTHEMIA

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JAK2V617F mutation has been reported in about 40-60% of Essential Thrombocythemia (ET) patients. However, little is known about specific molecular abnormalities of the hematopoietic stem cell compartment of ET according to JAK2 mutation. The aim of this study was to evaluate whether JAK2V617F mutation causes altered gene expression profile in the CD34+ stem/progenitor cell compartment of ET as compared to that of JAK2V617F-negative patients to identify differentially expressed genes. Total RNA from bone marrow CD34+ cells of 8 JAK2V617F-positive and 8 JAK2V617F-negative ET patients was extracted and two-cycle target labeling assays, as well as the Affymetrix HG-U133A GeneChip arrays hybridization, staining, and scanning, were performed, using Affymetrix standard protocols. Our results demonstrate that CD34+ stem/progenitor cells of a subset of ET patients harbor JAK2V617F mutation. Moreover, we show that the gene expression profile of ET is not significantly altered in JAK2V617F-positive CD34+ stem cells as compared to JAK2V617F-negative counterparts. By using Real-Time Quantitative RT-PCR, we also demonstrate that the expression of target genes of the JAK2/STAT pathway (BCL2L1, MYC, PIM1, SOCS1, SOCS2) is not significantly different among the two groups of patients. Consistently, when the pattern of gene expression identified in normal bone marrow CD34+ stem/progenitor cells was compared with that of ET patients (JAK2V617F-positive and negative), we found differentially expressed genes but we did not observe significant differences in expression for any of the target genes of the JAK-STAT pathway. Therefore, our findings suggest that JAK2V617F mutation has no significant influence on gene expression profile of stem/progenitor cells in ET. Supported in part by BolognAil

C007

DEVELOPMENT OF A REAL-TIME PCR ASSAYS FOR MPLW515L/K MUTATION WITH LNA-OLIGONUCLEOTIDE PROBES

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Acquired mutations in MPL (W515K or W515L) have been described in primary myelofibrosis (PMF) or essential thrombocythemia (ET). Direct sequencing or melting curve analysis, currently employed for genotyping, are cumbersome and have low sensitivity. We developed a real-time PCR (RT-PCR) assay for detection and quantification of MPLW515L/K mutation that is based on locked nucleic acid (LNA) fluorescent probes. LNA probes have increased thermal stability and hybridization specificity due to higher Tm, and were key for development of this assay since conventional TaqMan probes preliminary tested displayed very poor specificity. Genotyping was performed using granulocytic DNA; reference curves were prepared with cloned fragments of MPL containing 1 to 10^6 copies of un-mutated or mutated sequence. Efficiency of RT-PCR was almost absolute (r=0.98); using progressive dilutions of plasmid alleles, from 0.1% to 100% in a solution of un-mutated plasmid allele, we obtained a detection limit of at least 0.1% mutated allele in an un-mutated *Background.* This sensitivity level was attained using 40 ng of gDNA from diagnostic samples, as confirmed in assays using progressive dilutions of a 100% mutated patient in un-mutated gDNA. None of sixty control subjects presented MPLW515L/K mutation. The RT-PCR assay was validated by retrospectively analyzing a population of 217 patients with myelofibrosis, who had been previously genotyped using direct sequencing (Guglielmelli *et al.*, BJH 2007). Nineteen patients (8.7%) harbored MPLW515 mutation, of whom 10 (52.6%) had the W515L allele; in one case both W515L and W515K allele were detected by real-time PCR. In one case with positive RT-PCR using the W515K probe sequencing revealed a novel codon 515 mutation, W515A, due to a TGG>AGG transversion. By comparing results obtained with sequencing, no erroneous genotype attribution with RT-PCR was found, whereas one patient considered as un-mutated with sequencing analysis was found to harbor low W515L allele burden. This is a simple, sensitive and cost-effective procedure for large-scale screening of MPLW515L/K mutation in the work-up of patients suspected to have ET or PMF. It can also provide a quantitative estimate of mutant allele burden that might be useful for patient prognostication and for monitoring the response to therapy; the latter point is of relevance considering that novel targeted drugs against activated JAK/STAT pathway are already entering the therapeutic scenario.

C008

SILENCING OF SURVIVIN INDUCED BY A BCR-ABL/JAK2/STAT3 PATHWAY KILLES CML CELLS AND SENSITIZES IM-RESISTANT CLONES TO HYDROXYUREA

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The BCR-ABL oncoprotein of Chronic Myelogenous Leukemia (CML) displays constitutive tyrosine kinase activity and induces survivin expression at both the mRNA and protein level. High levels of survivin inhibit the apoptotic machinery of CML cells, contributing to the expansion of the leukemic clone. We report that, in murine and human CML cell lines, BCR-ABL-mediated up-regulation of survivin involves the JAK2/STAT3 pathway since silencing of either protein caused a consistent reduction of survivin. We also observed that survivin silencing significantly increased cell death in CML cells exposed to hydroxyurea but failed to restore Imatinib Mesylate (IM) sensitivity in IM-resistant cells, indicating that survivin expression lowers the sensitivity of CML cells to apoptotic stimuli but is not directly involved in the development of resistance to IM. However, down-regulation of survivin strongly increased HU-mediated killing of cells unresponsive to IM because of point mutations in the BCR-ABL kinase domain or amplification of the BCR-ABL gene. Finally, incubation of these IM-resistant cells with cell-permeable shepherdin, an inhibitor of heat shock protein 90 that reduces survivin expression, also enhanced HU-induced cell death. These results suggest that strategies aimed at reducing survivin levels increase the sensitivity of CML cells to HU, and may therefore represent a potential therapeutic option for CML patients regardless of their responsiveness to IM.

Myelodysplasia and Acute Myeloid Leukemia

C009

THE IRON CHELATOR DEFERASIROX IS A STRONG INHIBITHOR OF NF-KB ACTIVITY IN MDS CELLS AND IN HL60 AND K562 CELL LINES, THIS EFFECT IS NOT SHARED BY THE OTHER CHELATORS

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Background. Iron overload is a critical issue for low risk MDS patients with a long tranfusional history. Recently a once daily oral chelator Deferasirox became available for treatment of secondary hemosiderosis. Iron chelation is an independent prognostic factor for survival in MDS but can also improve haemoglobin level in some cases. It was demonstrated that NF-KB is abnormally activated in MDS blast cells, even in the absence of iron overload. *Aim.* to compare the effects of the commercially available iron chelators on NF-KB activity in MDS and to identify a possible mechanism responsible for the observed reduced transfusion requirement during chelation therapy. *Methods.* 40 PB samples were collected from MDS patients: 18 RA, 14 RAEB, and 8 s-AML. 30 of them presented iron overload (by SQUID biomagnetic liver susceptometry) and high serum ferritin level >1000 ng/mL. 10 samples were collected before starting transfusion therapy. MNC cells were incubated with 50 microM Deferasirox for 3 hrs. K562 and HL60 cells were analyzed as controls and were incubated with Deferasirox, Deferiprone and Deferioxamine. NF-KB activity was evaluated using both EMSA and ELISA methods. *Results.* We detected an increased activation of NF-KB as compared to healthy subjects in 6 RA, 12 RAEB, in all the s-AML PB samples and in cell lines. No significant difference was detected in NF-KB activity comparing patients with or without iron overload (p=0,5). The percentage of samples presenting NF-KB activity increases during disease progression being higher in RAEB and s-AML as compared to RA (p=0,003). Among patients with increased NF-KB (n=14) the incubation with Deferasirox induced a significant reduction of NF-KB activity (p=0,0002). No difference was found in NF-KB inhibition comparing patients with or without iron overload. When HL60 and K562 cells were incubated with the different chelators only Deferasirox was able to reduce NF-KB activity. *Discussion.* NF-KB is abnormally activated in MDS patients and this is not related to iron overload being present in patients with normal serum ferritin levels and in cell lines. Deferasirox but not Deferioxamine or Deferiprone acts as a potent NF-KB inhibitor. This peculiar behaviour of the drug seems to be independent from the reduction of iron storage induced by chelation and could explain the early improvement of hemoglobin levels observed in some patients under Deferasirox chelation.

C010

PI-PLC-BETA1 MONO-ALLELIC DELETION IN MYELODYSPLASTIC SYNDROMES

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Nuclear PI-PLCbeta1 appears as one of the main players of growth and differentiation processes, not only in normal but also in pathological conditions. Myelodysplastic syndromes (MDS) are a group of hematological disorders characterized by progressive cytopenia and increased tendency to acute myeloid leukemia (AML) transformation. The mechanisms underlying the evolution into AML are not completely understood. However, recent studies hint at the likelihood that an aberrant regulation of intracellular signalling pathways is involved in the pathogenesis of MDS, mainly through PI-PLCbeta1 and its relationship with the PI3K/Akt axis. Indeed, we demonstrated not only an activation of the Akt/mTOR survival pathway in high-risk MDS, but also an inverse correlation between the expression of p-Akt and PI-PLCbeta1, which could lead to an imbalance in cell proliferation and apoptosis. By using FISH analysis we have previously evidenced that, in MDS patients with normal karyotype and a fatal outcome, the PI-PLCbeta1 gene undergoes a mono-allelic and interstitial deletion, hinting at a direct involvement of PI-PLCbeta1 in the progression into AML. In the present study we evaluated 80 MDS cases. FISH analysis was performed on each patient at the time of diagnosis, to determine the presence of PI-PLCbeta1, PI-PLCbeta4 and PI-PLCgamma. As a control, we evaluated the status of either the 20p or the 20q sub-telomeric region, where these genes are mapped. The deletion is present mainly in high-risk MDS; whilst low-risk MDS, usually having a much better outcome, show this genetic anomaly in a very little number of cases. In particular, 32/80 (40%) cases, either with normal or altered karyotype, low-risk or high-risk MDS, showed the mono-allelic deletion of the PI-PLCbeta1 gene, while both PI-PLCbeta4 and the 20p sub-telomeric region were normal in all the patients analyzed. Interestingly, the patients bearing the mono-allelic deletion of PI-PLCbeta1 had a worse outcome, as compared with patients having both alleles, since most of them evolved into AML. As for PI-PLCgama, preliminary data indicate that this gene is not specifically linked to a worse outcome. Taken together, our results suggest the possible involvement of PI-PLCbeta1 in the progression of MDS into AML and hint at the identification of a new group among high-risk MDS patients. However, further investigations are needed to fully understand the molecular mechanisms underlying the progression of MDS into AML.

C011

MOLECULAR ANALYSIS OF T(15;17) GENOMIC BREAKPOINTS IN SECONDARY ACUTE PROMYELOCYTIC LEUKEMIA ARISING AFTER TREATMENT OF MULTIPLE SCLEROSIS

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Therapy-related acute promyelocytic leukemia (t-APL) with t(15;17) translocation is a well-recognized complication of cancer treatment with agents targeting topoisomerase II (topoII). However, cases are emerging following mitoxantrone (MTZ) therapy for multiple sclerosis (MS). We characterized at the genomic level the PML and RARA breakpoints of 15 patients who developed APL on a background of MS, including 13 who received MTZ for MS. A long range nested PCR strategy followed by direct sequencing was adopted. Furthermore, we used MTZ induced topoII alpha mediated *in vitro* DNA cleavage assays to elucidate the mechanisms underlying the formation of the t(15;17) in this setting. Analysis of 13 cases of MTZ related t-APL in MS patients revealed an altered distribution of chromosome 15 breakpoints compared to de novo APL, biased towards disruption within PML intron 6 (12/13, 92% *vs.* 622/1022, 61%: p=0.021). Despite this intron spanning ~1kb, the breakpoint in 5 MTZ treated patients fell within an 8bp region (1482-9) corresponding to the "hotspot" previously reported in t-APL complicating MTZ containing breast cancer therapy. Another shared breakpoint was identified within ~17kb RARA intron 2 involving two t-APL cases arising after MTZ treatment for MS and breast cancer, respectively. Analysis of PML and RARA genomic breakpoints in functional assays in 4 cases, including the shared RARA intron 2 breakpoint at 14444-48, confirmed each to be preferential sites of topoII alpha mediated DNA cleavage in the presence of MTZ. Based upon sequence analysis of PML-

RARA and reciprocal RARA-PML genomic breakpoints, the location of functional topoII alpha cleavage sites in the vicinity of the breakpoints, and known mechanisms by which topoII induces double-strand breaks in DNA and their subsequent repair, it was possible to generate models as to how the t(15;17) chromosomal translocation could have been formed (Figure 1). This study lends further support to the presence of preferential sites of DNA damage induced by MTZ within PML intron 6, and suggests the existence of a further "hotspot" at the distal end of RARA intron 2. The susceptibility of these regions of the PML and RARA loci to topoII alpha mediated cleavage by MTZ may underlie the propensity to develop this particular subtype of AML following exposure to this agent. Further studies are warranted to investigate whether MS patients have a particular predisposition to the development of secondary APL.

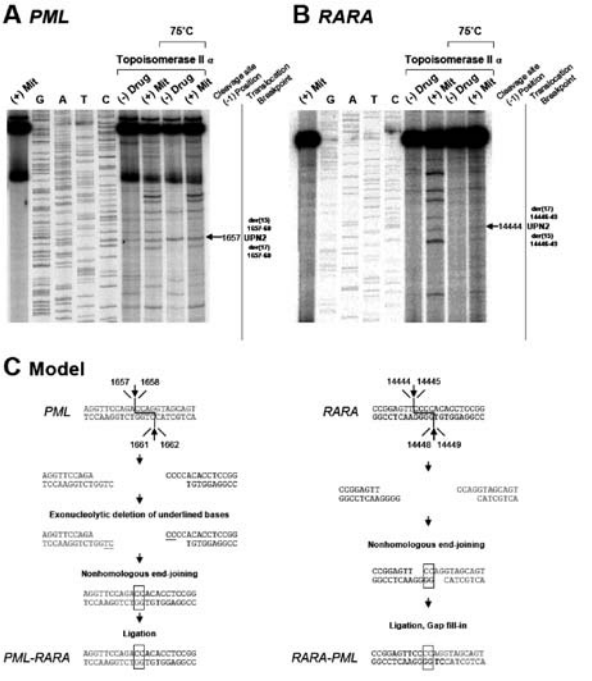

Figure 1.

C012

IMMUNOSUPPRESSION BY ALEMTUZUMAB FOR THE TREATMENT OF BONE MARROW FAILURE SYNDROMES

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Acquired aplastic anemia (AA) is an immune-mediated marrow failure syndrome affecting all hematopoietic lineages; a similar pathophysiology is also postulated in single-lineage failures, such as pure red cell aplasia (PRCA) or agranulocytosis (AGR). We investigated an experimental immunosuppression (IS) regimen based on the anti-CD52 antibody alemtuzumab (MabCampath®, ALE) in a phase II/III study. Twenty-one patients (8 AA, 10 PRCA and 3 AGR) were enrolled, who received ALE subcutaneously in consecutive days (3-10-30-30-[30] mg, total dose 103 for AA and 73 for PRCA and AGR) with adequate premedication. All patients started oral low dose cyclosporine A (1 mg/kg) on day 7; an intensive anti-infectious prophylaxis was exploited, which included oral valgancyclovir and cotrimoxazol. All patients completed the treatment with no relevant injection-related side effect (with exception of fever and/or rash in some cases), nor significant clinical or laboratory abnormality. A complete lympho-ablation was observed in all patients within 2-3 days, which persisted for several weeks; transient worsening of neutropenia and/or thrombocytopenia were observed in some patients. The median follow-up was 12 months, with a cumulative observation of 200 patient-months; there were 3 deaths (all related to progression of concomitant independent diseases). Response was assessed in patients with follow up longer than 3 months: among the 7 AAs, we had 4 CR, 1 PR, 2 NR. In the 6 PRCAs, there were 4 CR and 2 NR (1 progressing to AREB); 2 out of 3 AGRs obtained CR (the third reclassified as MDS, due to progression to AREB). Among responding patients, relapses were quite frequent: 3/5 SAAs, 4/4 PRCAs and 1/2 AGR, which were successfully treated by additional ALE (as single shoots or complete courses). The safety profile was excellent, given that infectious events were irrelevant: clinically, 1 VZV with shingles, 2 HSV and 1 flu have been recorded, all resolving quickly. No CMV or EBV disease was observed, even if 3 border-line CMV reactivations were documented, promptly resolved by preemptive valganciclovir. One HBV reactivation without hepatitis required lamivudine. Immune reconstitution was delayed up to several months, especially affecting the $CD4^+$ compartment; this was also due to additional ALE needed to sustain the hematological response. In conclusion, subcutaneous ALE is a safe IS regimen showing an excellent response rate and easy retreatment in immune-mediated marrow failure syndromes.

C013

THE COMBINED ANALYSIS OF FLOW-CYTOMETRIC MINIMAL RESIDUAL DISEASE DETERMINATION AND MUTATIONAL STATUS OF FLT3 AND NPM IMPROVES THE PROGNOSTIC STRATIFICATION OF ADULT AML PATIENTS WITH NORMAL KARYOTYPE

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In a remarkable proportion of adult AML patients (40-50%), no clonal abnormalities are found on standard cytogenetic analysis. In this group of patients, mutations in several genes (FLT3, NPM, BAALC, CBPA) have been described, allowing to discriminate subgroups with distinct clinical outcome. Although these molecular signatures allow a reliable upfront prognostic evaluation in many patients (up to 60% for NPM mutations) the clinical impact of detecting this gene alterations for monitoring minimal residual disease (MRD) in the post-remission phase is still under investigation. To this purpose, we investigated a group of 80 normal karyotype AML patients entered into the EORTC/GIMEMA protocols AML10/AML12 (age <61yrs) or AML13/AML15/AML17 (age >61 yrs), and compared the prognostic information derived from the up-front determination of FLT3 and NPM mutational status to the prognostic role of MRD as determined by multiparametric flow-cytometry (MPFC) in the post-consolidation phase. By applying the maximally selected log-rank statistics, the threshold discriminating MRD negative from positive cases was set at 3.5×10^4 residual leukemic cells, a level that distinguished, at the post-consolidation time-point, two groups of patients with distinct prognosis. Among these 80 patients, In post-consolidation MRD status was significantly associated with prognosis, with MRD negative patients showing a better prognosis with 63% and 75% a 5-years OS and RFS, respectively, vs. OS and RFS <20% in MRD positive (p=0.006). Sixty out of 80 patients were studied for FLT3 mutational status and in 12 (20%) a FLT3-ITD mutation was detected; NPM1 mutation was studied in 63 out of 80 patients and in 22 (35%) a mutated status was discovered. Patients without FLT3-ITD had a better prognosis (5-years OS/RFS 41% 31%, respectively, *vs.* OS/RFS <15%, p=0.038).

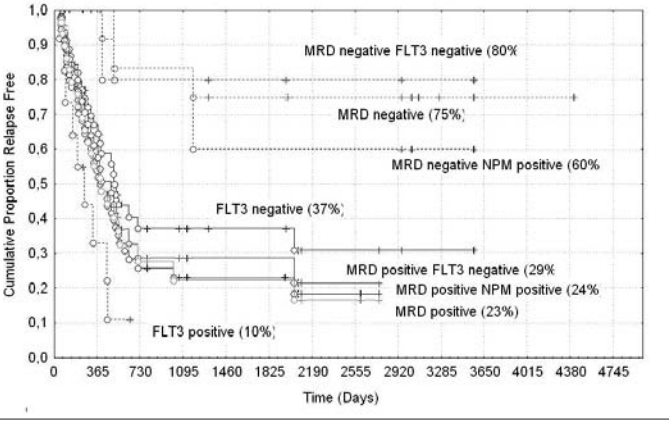

Figure 1. RFS analysis in 80 adult AML patients.

The combined analysis of FLT3 and NPM revealed a favorable impact of NPM mutations on OS and EFS only in the group of patients without

FLT3 mutations. Finally, when molecular and MPFC analyses were integrated, we observed that: 1) MRD positive patients did as worse as FLT3 positive patients; 2) FLT3 negative MRD positive patients shared the same dismal prognosis of FLT3 positive patients (Figure 1). Similarly, post-consolidation MRD status divided in two discrete groups with discrete outcome even NPM positive patients , (5-years RFS 60% *vs.* 24%, p=0.018), with those NPM positive MRD positive belonging to the poor risk category. In multivariate analysis, MRD status post-consolidation and FLT3 confirmed their prognostic role on RFS (p=0.003 and p=0.032, respectively). In conclusion, in patients with normal karyotype combined evaluation of MRD determination and FLT3/NPM allows the prognostic assessment of AML to be refined thus indicating more appropriate therapeutic strategies.

C014

IDENTIFICATION OF SOMATIC JAK3 MUTATIONS IN ACUTE MYELOID LEUKEMIA

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The activating Janus kinase 2 mutation (JAK2V617F) is seen in most Polycythemia Vera (PV) as well as in one third of Essential Thrombocythemia and Primary Myelofibrosis (PMF) patients. Notably, additional JAK2 and MPL (thrombopoietin receptor) mutations have also been reported, some of which can induce a PV-(JAK2) or PMF-like (MPL) phenotype in mice. Thus JAK-STAT pathway appears to play a central pathogenetic role in myeloid malignancies and JAK tailored therapies may represent novel modalities for the treatment of these processes. The Janus kinase genes encode non-receptor tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) and gain-of-function (GOF) mutations have been described for JAK2 and JAK3. Rare JAK3 GOF mutations (JAK3A572V, JAK3V722I [JH2 domain] and JAK3P132T [JH6 domain]) have been recently described in primary and cell lines derived from patients with acute megakaryocytic leukemia (AMKL). Here, we sought to further evaluate the frequency and the possible leukemogenesis role of JAK3 mutations in de novo acute myeloid leukemia (AML). We sequenced the JAK3 JH2 domain, (including A572V and V722I) and the exon 3 of the JH6 domain spanning the amino acid site P132T, of 134 de novo AML and 191 control DNA samples. All AML and control DNA were wild-type for the A572V substitution (119/119). Moreover, we found two other putative activating substitutions [V722I (2/134) or P132T (1/119)] in AML samples, however at the same frequency observed in our control set, suggesting that these putative mutations may simply represent Single Nucleotide Polymorphism (SNP, V722I 5% and P132T 1%). Interestingly, in a single AML-M7 (1 of 28), a novel homozygous mutation at 132 position, which leads to a transversion of C to G (proline at 132 to alanine, P132A) was documented. To examine the pathogenetic role of JAK3 mutations, we transfected the wild type (JAK3WT) and JAK3V722I, JAK3P132A, JAK3A572V, into IL-3 dependent Ba/F3 cells and cell growth was measured after IL-3 withdrawal. Only JAK3A572V positive Ba/F3 cells grew in the absence of IL-3. These findings were also confirmed using NIH 3T3 transfection/focus formation assays and murine xenograft models, *in vivo*. Overall, our results indicate that only JAK3A572V has a transforming potential. Thus additional studies may be required to dissect the precise pathogenetic contribution of JAK3 mutations in myeloid proliferative disorders.

C015

A ONE-MUTATION MATHEMATICAL MODEL CAN EXPLAIN THE AGE INCIDENCE OF AML WITH MUTATED NUCLEOPHOSMIN

Liso A, Castiglione F, Cappuccio A, Stracci F,. Schlenk RF, Amadori S, Thiede C, Schnittger S, Valk PJM, Döhner K, Martelli MF, Schaich M, Krauter J, Ganser A, Martelli MP, Löwenberg B, Haferlach T, Ehninger G, Mandelli F, Döhner H, Michor F, Falini B

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Acute myeloid leukemia (AML) with mutated NPM1 gene is a new disease entity which accounts for about one-third of all adult AML. Experimental evidence of the oncogenic potential of the nucleophosmin mutant is, however, still lacking, and it is unclear whether other genetic lesion(s), e.g. FLT3 internal tandem duplication (ITD), cooperate with NPM1 mutations in AML development. We collected data sets at five major European Institutions: the Laboratory of Cytogenetic and Molecular Diagnostic, University Hospital Ulm, representing the German-Austrian AML study Group ; the Laboratory of Hemopathology, Institute of Hematology, University of Perugia, representing GIMEMA group; the Laboratory for Molecular Diagnostics, University Hospital Carl Gustav Carus, Dresden, Germany, representing the Deutsche Studieninitiative Leukämie; the Munich Leukemia Laboratory, and the Department of Hematology, Erasmus University Medical Center. We determined the age-specific incidence of AML with mutated NPM1 (a total of 1444 cases) for each country. We then adapted a previously designed mathematical model of hematopoietic tumorigenesis to analyze the age incidence of AML with mutated NPM1. Finally, we confirmed the predictions of equations with direct computer simulation of the stochastic process. Linear regression of the curves representing age-specific rates of diagnosis per year showed similar slopes of about 4 on a doubly logarithmic scale. The one-mutation model generated slopes similar to real age-specific incidence curves from patients (Figure 1) from Germany, The Netherlands, and Italy. We then determined whether the age incidence of NPM1-mutated AMLs with FLT3-ITD differs from cases with wild-type FLT3. No significant difference emerged in the slopes of FLT3-ITD-positive and -negative AML with mutated NPM1. The model fits the $NPMc^+$ AML age-specific incidence curve for plausible parameter choices supporting the hypothesis that a single genetic event, or two sinchronous events, are sufficient to cause this type of leukemia. No difference can be detected between the slopes of the age specific incidence of FLT3-ITD-positive and -negative $NPMc^+$ AML, supporting the view that $NPMc^+$ AML is a homogeneous group irrespective of the FLT3 mutational status. Since NPM1 mutations are associated with haploinsufficiency of wild-type NPM in leukemic cells, an attractive hypothesis would be that the two alterations act together to cause $NPMc^+$ AML.

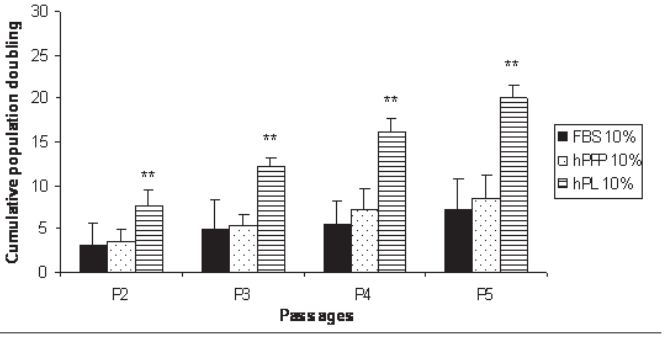

Figure 1. Expansion capacity of AT-MSCs cultured in FBS, hPPP or hPL. Data are expressed as mean±SD of five samples of each condition (, p<0.01).**

C016

PROLIFERATIVE AND APOPTOTIC SIGNALLING IN BONE MARROW CELL SUBPOPULATIONS OF MYELODYSPLASTIC SYNDROME PATIENTS

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Myelodysplastic syndromes (MDS) are heterogeneous clonal diseases characterised by cytopenias as a result of ineffective hemopoiesis. Development of effective treatments has been impaired also by limited insights into MDS pathogenesis. Moreover, little is known about signal transduction pathways altered MDS cells. We have devised a method of analysis of signal transduction pathways using multiparameter flow-cytometry (FacsCanto cytometer Becton Dickinson (BD)), allowing rapid and specific separate evaluation of specific cellular subpopulations in MDS bone marrow samples. We used the (BD) Phosflow reagents. We obtained optimal results by first fixing cells with formaldehyde (BD Cytofix buffer) for 10 minutes at 37°C, then permeabilizing with methanol (BD Perm BufferIII) for 30 minutes on ice and staining 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 with 6 colors. We studied basal phosphorylation of ERK1/2 and p38 MAP kinases, basal 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 $CD71^+$ obtained from bone marrow aspirates of 40 MDS patients versus normal bone marrow cells from healthy donors. Moreover, we analysed the effects of erythropoietin (EPO) and granulocyte colony stimulating factor (G-CSF) stimulation, and their combination on ERK1/2, p38, STAT5 and caspase-3 activation in $CD34^+$, $CD45^+$ and $CD71^+$ cellular subsets in MDS versus normal donor bone marrow cells. Basal activation of MAPK phospho-proteins, STAT5 and cleaved caspase-3 varies among primary MDS cells and it is different among the cellular subsets. EPO stimulation fails to induce Stat5 activation in erythropoietic cells in MDS while is active in normal cells. G-CSF is activating signal transduction mostly in $CD34^+$ MDS cells. We conclude that signal transduction pathways may be analysed in MDS bone marrow cells distinguishing specific cellular subsets, by flow-cytometry techniques. This approach could represent a feasible and rapid method to apply in large scale studies.

Molecular and Cell Biology, Normal and Malignant Hematopoiesis

C017

ANGIOGENESIS AND MALIGNANCIES OF HEMOPOIETIC ORIGIN: ROLE OF GENETIC POLYMORPHISMS IN THE VASCULAR ENDOTHELIAL GROWTH FACTOR GENE IN THE DEVELOPMENT OF KAPOSI'S SARCOMA (KS)

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Introduction. Many experimental evidences highlighted the role of VEGF in the pathogenesis of hematological malignancies like multiple myeloma, ALL, AML and CML. VEGF influences the microenvironment through autocrine/paracrine loops promoting survival, proliferation and migration of leukemic cells. Circulating levels of VEGF could be considered a marker of disease progression and therapeutic response. Human herpesvirus 8 (HHV8) associated-KS is a vascular tumor of hemopoietic origin, characterized by deregulated angiogenesis, extravasation of inflammatory cells and proliferation of neoplastic spindle shaped cells. Recent studies have shown that KS derives from a $CD34^+$ KDR^+ lymphoendothelial progenitor. VEGF promotes spindle cell proliferation and inflammation within early KS lesions. Many of the polymorphisms in the VEGF gene are able to influence expression levels. We sought to determine whether those genetic variations could predispose to the development of KS. *Results.* We evaluated known genetic polymorphisms within the promoter, 5' UTR and 3' UTR in the VEGF gene in 81 subjects affected by KS (46 classical, 35 post transplant) and 295 healthy controls (150 blood donors, 145 transplant recipients). Five polymorphisms were statistically associated with a higher risk of developing KS: -1512 18 nt insertion, -460 allele C, -152 allele A, -116 allele A, +405 allele G. Because of their short distance, these SNPs can be inherited together; we therefore searched for haplotypes that could correlate with KS development. Haplotypes -1512wt/-460T/-152A/-116A/+405G and -1512 insertion/-460C/-152A/-116A/+405G were associated with a higher risk of classical KS and post transplant KS, respectively. We also quantified VEGF serum levels in KS cases and controls; circulating VEGF correlated neither with the pathology, nor with single polymorphisms, nor with haplotypes. *Conclusions.* Our findings show, for the first time, the existence of a genetic predisposition to classical and post transplant KS, suggesting that a particular genetic profile might account for the local, i.e. in lesions, rather than systemic, levels of VEGF. Genetic screening for VEGF polymorphisms may represent an important tool in identifying HHV8 infected individuals at risk of KS, especially in the setting of transplantation. The present data might be the basis for predisposition studies in other hematological diseases, holding angiogenesis as a key feature.

C018

INVOLVEMENT OF CD34+ HEMATOPOIETIC STEM CELLS IN MDS/AML WITH RARE TRISOMIES

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Background. Although trisomy 8 (+8) is one of the most frequent isolated aberrations in myelodysplastic syndromes (MDS) and acute myeloid leukaemia (AML), rare trisomies of other chromosomes do recur. Attempts to identify the cell of origin in MDS/AML with +8 showed the myeloid compartment, i.e. $CD13^+$ and $CD33^+$ cells and hematopoietic stem cell pool ($CD34^+CD38^-$Thy-1^+ cells) belonged to the abnormal clone. No information on cell lineage affiliation are available for other rare isolated trisomies. *Aim*. To investigate rare isolated trisomies in patients with MDS/AML using FICTION (Fluorescence Immunophenotype and Interphase Cytogenetics as a Tool for Investigations of Neoplasms), which combines simultaneous immunophenotyping and interphase FISH on bone marrow cells. *Patients*. We studied one

AML with trisomy 10, two MDS with trisomy 14 and 11, respectively, and two MDS with trisomy 15. *Fiction.* Mononuclear bone marrow cells were investigated using anti-CD34, anti-CD133, anti-CD33, anti-CD13, anti-CD14, anti-glycophorin A, anti-CD19, anti-CD20, anti-CD3 and anti-CD7 monoclonal antibodies. The panel of DNA clones we applied were: centromeric probes for chromosomes 10 and 15; "LSI MLL break-apart (Vysis, Olympus, Italia) for chromosome 11q23; and "LSI IgH break-apart (Vysis, Olympus, Italia) for chromosome 14q32. At least 32 cells (range 32-133) were analysed for each antibody. *Results.* In all cases hematopoietic precursors ($CD34^+$ and $CD133^+$) and myelomonocytic cells ($CD33^+$, $CD14^+$, $CD13^+$) belonged to the abnormal clone. Erythroid cells (Glycophorin A^+) were also trisomic in the MDS patient with trisomy 14 and in two MDS with trisomy 15. B-lymphoid ($CD20^+$) cells were also involved in MDS with +14 and T-lymphoid ($CD7^+$) cells in the MDS with +11. *Discussion.* In MDS/AML with isolated, rare, recurrent trisomies, totipotent hematopoietic stem cells and myelo-monocytic cells always belonged to the cytogenetically abnormal clone while B and T lymphocytes were involved only sporadically. These findings suggest that chromosomes gains occur in a pluripotent stem cell which differentiates towards myeloid and lymphoid lineages. These new insights into the biology characterization of MDS/AML with uncommon trisomies are similar to previous reports on MDS/AML with del(20q) and monosomy 7. *Supported by Miur-Prin; Fondazione Cassa di Risparmio; Associazione Sergio Luciani, Fabriano, Italy; AULL, Associazione Umbra Leucemie e Linfomi, Perugia, Italy*

C019

ROLE OF CD34 ANTIGEN IN MYELOID DIFFERENTIATION OF HUMAN HEMATOPOIETIC PROGENITOR CELLS

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CD34 is a highly glycosylated transmembrane protein, strongly expressed on hematopoietic stem/progenitor cells (HSPCs); despite its importance as a marker of HSPCs, its function is still poorly understood. Several studies suggest that CD34 may play a role in cell adhesion and signal transduction on hematopoietic stem/progenitor cells or mediate the interaction between HSPCs and stromal lectins in the bone marrow (BM). To characterize the function of CD34 antigen on human HSPCs, we examined both by small interfering RNAs (siRNAs) and by retroviral-mediated over-expression, the role of CD34 antigen in the regulation of hematopoietic stem/progenitor cell lineage commitment. Here, we demonstrate that the expression of CD34 antigen is able to affect the differentiation capacity of human HSPCs, in particular clonogenic assay and serum-free liquid culture show that the early down-regulation of CD34 enhances granulocytic and megakaryocitic differentiation at the expense of the erythroid one. In agreement with these results, microarrays analysis reveals that siRNA-mediated CD34 gene silencing in $CD34^+$ HSPCs induces the up-regulation of genes involved in granulocytic and megakaryocytic commitment and the down-regulation of the erythroid genes. In order to confirm the results obtained by RNA interference, we over-expressed the human CD34 cDNA in human $CD34^+$ progenitor cells by retroviral gene transfer. CD34 transduced cells show a remarkable increase of erythroid progenitors (BFU-E and CFU-E) and a dramatic decrease of granulocytic (CFU-G) progenitors as evaluated by clonogenic assay. Furthermore, immune phenotype analysis indicates that granulocytic differentiation markers (CD66b and MPO) in CD34 transduced cells exhibit a decreased expression; on the contrary, the expression of erythroid marker GPA is up-regulated. Finally, microarray gene expression profile shows an increased expression of erythroid genes in CD34 transduced cells. Moreover, we find many markers of granulocytic differentiation such as neutrophil and eosinophil granule proteins among down-regulated genes. Together, these data indicate that the CD34 molecule promotes the differentiation of $CD34^+$ hematopoietic progenitors towards the erythroid lineage, which is achieved, at least in part, at the expense of other maturation lineages such as the granulocytic and megakaryocytic ones.

C020

ARSENIC TRIOXIDE IN COMBINATION WITH BORTEZOMIB EXERTS SIGNIFICANT SYNERGISTIC ANTI-PROLIFERATIVE AND PRO-APOPTOTIC EFFECTS IN HL60 CELL LINE: A GENE EXPRESSION STUDY

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Bortezomib in combination with arsenic trioxide (ATO) had significant antitumor effect in nude mice bearing HL60 cell xenografts (Lin, 2007). In line with these results, we further clarified anti-proliferative and pro-apoptotic effects by gene expression assays. Cell viability was assessed by MTT; apoptosis by the AnnexinV/propidium. ROS production was evaluated by dihydrorhodamine 123. Samples were hybridized on Whole Human Genome Microarray (Agilent) and analyzed for network and gene ontology by Ingenuity Pathway Analysis software. Results have been confirmed by using the TaqMan Low Density Array Human Apoptosis Panel. A synergistic inhibition of cell growth was observed after 24h-combinated treatment (34% of cell death *vs.* 14% of ATO, 13% of bortezomib, and 5% in untreated cells CI=0.86). Moreover, co-treatment significantly increased the apoptotic rate up to 58% (CI=0.75, Figure 1). This pro-apoptotic effect was confirmed by the cell cycle analysis: both drugs blocked cell cycle in the G2-M phase, but the sub-G1 subpopulation significantly increased after the co-incubation, concomitantly to the alteration of mitochondrial membrane permeability, as showed by TMRE assays. A higher ROS production was observed after arsenic treatment, and the co-exposure to bortezomib did not increase this phenomenon. To investigate the role of caspases, Z-LEHD-FMK and Ac-DEVD-CHO (inhibiting caspase 9 and caspases 3 and 7, respectively) were tested. Caspase 9 inhibitor reduced arsenic lethality, whereas failed to block the toxicity of the association. On the other hand, the caspases 3 and 7 inhibitor significantly reduced the toxicity exerted by ATO and bortezomib. Moreover, the association significantly increased the expression of the FAS receptor and DR5. Finally, EMSA assay showed that both drugs alone and in combination were able to inhibit NF-kB. Gene expression assays confirmed these results, showing a significant down-regulation of BIRC1, and up-regulation of CARD9, DEDD, FAS, FADD, DIABLO, TNFSF10; TNFSF1B, NFKBIA, NFKBIB, NFKBIE. The co-exposure down-regulated MLL, a potent leukemogenic oncoprotein, HSP90 and HSP70, overexpressed in poor-prognosis acute leukemias, and HMOX1, responsible for the resistance of AML cells to the TNF. Other genes significantly down-regulated were ICAM1, cJUN, WT1, and PMAP1. In summary, biological results and gene expression assays suggest the possible combinated use of arsenic and bortezomib in the treatment of acute myeloid leukemia.

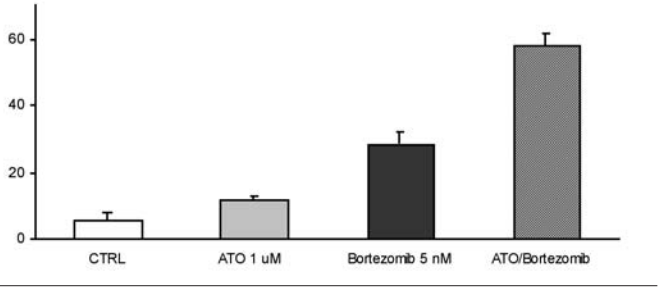

Figure 1.

C021

A NOVEL FUSION 5'AFF3/3'BCL2 ORIGINATED FROM A t(2;18)(Q11.2-Q21.33) TRANSLOCATION IN FOLLICULAR LYMPHOMA

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Follicular lymphoma is the second most frequent type of non-Hodgkin's lymphoma in adults. The basic molecular defect consists in the t(14;18)(q32;q21) translocation, juxtaposing the B-cell lymphoma protein 2 gene BCL2 to the immunoglobulin heavy chain locus IGH@, and leading to the antiapoptotic BCL2 protein over-production. Variations in the t(14;18) are rare and can be classified into two categories: i) simple variants, involving chromosomes 18 and 2, or 22, in which the fusion partner of the BCL2 is the light-chain IGK@ or IGL@; ii) complex variant translocations, occurring among chromosomes 14, 18, and other chromosomes. We report a follicular lymphoma case showing BCL2 overexpression, detected by immunohistochemistry and Real-Time Quantitative PCR, consequently to the formation of a novel fusion gene between the 5' of the lymphoid nuclear transcriptional activator gene AFF3 at 2q11.2, and the 3' of BCL2. The present case shows evidence, for the first time, of BCL2 overexpression consequently to the fusion of BCL2 to a non-IG partner locus.

C022

EVI1 IMPAIRS MYELOID DIFFERENTIATION BY DEREGULATION OF PU.1 FUNCTION

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EVI1 is an oncogene associated with myelodysplastic syndrome (MDS) and myeloid leukemia. We have developed a mouse model showing that constitutive expression of EVI1 in bone marrow impairs cells differentiation leading to severe pancytopenia resulting in MDS. It was reported that EVI1 blocks the G-CSF-induced differentiation of a myeloid cell line. We demonstrated that EVI1 alters the myeloid maturation of bone marrow progenitors and increases the number of immature myeloid elements *in vitro*. In this work we show that EVI1 represses a subset of genes, which are normally activated during the myeloid differentiation. These genes are under the control of the transcription factor PU.1. We demonstrated that EVI1 interacts with PU.1 and represses the PU.1-dependent activation of a myeloid promoter. We further show that EVI1 does not inhibit PU.1 binding to DNA, but blocks its association with the co-activator c-Jun. These results suggest that EVI1 alters the regulation of the myeloid transcription factor PU.1 and therefore impairs a proper differentiation of the myeloid elements. We identify an EVI1 point mutant unable to bind PU.1 that restores the activation of a PU.1-regulated promoter and allows a normal differentiation of the BM progenitors *in vitro*.

C023

IN THE HUMAN GENOME, ONLY FEW GENES CAN BE A TARGET FOR DUPLICATION ENCODING A NUCLEAR LOCALIZATION SIGNAL

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Tandem repeats, are frequent in mammalian genomes, and 8% of them lie in coding sequences. In fact the majority of short DNA insertions in humans are tandem duplications of directly adjacent sequence segments with conserved polarity. Repeat instability has been estimated as 100 000 times higher than that of point mutations. Within coding regions, this mechanism might produce frequent polymorphisms, translating into the contraction or expansion of amino-acid motifs. Indeed, duplications are likely to constitute the predominant process for rapid generation of new genetic material and function on smaller scales. Repeat instability is also a form of mutation that is linked to more than 40 neurological, neurodegenerative and neuromuscular disorders. Importantly, we first demonstrated Nucleophosmin (NPM1) mutations, occur in about 30% of adult acute myeloid leukemia (AML) that show distinct biological and clinical features. About 80% of NPM1-mutated AML bear a tandem duplication of a TCTG tetranucleotide at positions 956-959 of NPM1. Cytoplasmic NPM accumulation in leukemic cells bearing NPM1 mutation is caused by changes of tryptophan(s) 288 and 290 and creation of a new nuclear export signal (NES) motif at the C-terminus of the NPM mutant. NPM1 mutation appears to be a unique genetic event that is specific to AML. DNA repeat expansion mutations are dynamic and ongoing within tissues and across generations. Here we propose an innovative approach aimed at predicting the consequences upon generation of tandem repaeats in the coding human genome. In particular, we explored hypothetical duplication events generating known amino-acid motifs (other than NES) in the human genome. To this end we designed a computational approach based on a customized software. In particular, our computational analysis was restricted to motifs that contain amino acids both necessary and sufficient for nuclear import (Nuclear Localization Signals or NLS). After retrotranslation, each signal was processed in a search for tandem duplications with a period of 4-8 nucleotides as well as exact multiple duplications. When a duplication was identified, its last repeat was deleted and its DNA sequences were matched against human genome coding sequences. Our findings so far strongly suggest that, out of the entire human genome, only few genes fulfil the requirements for an ideal site for duplication encoding for a NLS motif. The results of this computational analysis will be discussed.

C024

TEL-AML1 DYSREGULATES THE TGFBETA PATHWAY: A BASIS FOR PRE-LEUKAEMIC STEM CELL SELECTION

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TEL-AML1 fusion is the most frequent, defined genetic abnormality in paediatric cancer and is usually an early or initiating and pre-natal event in childhood acute lymphoblastic leukaemia (ALL). Transformation results in the generation of a persistent pre-leukaemic clone, which converts to frank ALL post-natally (at 1-15 years) following the acquisition of secondary genetic alterations. The mechanism by which the transcriptional dysregulation imposed by TEL-AML1 impacts on the pre-leukaemic phenotype and disease natural history is unknown. Using regulated expression of TEL-AML1, we show that, in murine progenitor cells, this protein blocks the TGFbeta response pathway including the critical regulator of cell cycle inhibition, p27. This inhibition facilitates the selective expansion of otherwise more slowly expanding TEL-AML1 progenitor cells. The mechanism of action of TEL-AML1 appears to operate downstream of SMAD2/3 phosphorylation (by TGFbeta signalling) and involves binding of a transcriptional repressor complex to TGFbeta target genes. In transgenic TEL-AML1 mice, we show that early pro-B cells (but not pre-B cells) are selectively expanded and appear less sensitive to the inhibitory effects of TGFbeta than equivalent wild-type cells. Finally, we show that expression of TEL-AML1 in human cord blood progenitor cells leads to the expansion of a candidate pre-leukaemic stem cell population with an early B lineage phenotype ($CD34^+/CD38^-/CD19^+$) that has a selective growth advantage in the presence of TGFbeta.

Myeloma and Hemostasis Diseases

C025

ABSENCE OF THE JAK2 EXON 12 MUTATIONS IN PATIENTS WITH SPLANCHNIC VENOUS THROMBOSIS AND WITHOUT OVERT CHRONIC MYELOPROLIFERATIVE DISORDERS

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Background. Thrombosis of major abdominal veins has been reported in 5% to 10% of the patients with polycythemia vera (PV) or essential thrombocythemia (ET). Conversely, molecular hallmarks of chronic myeloproliferative disorders (CMD) can be recognized in a substantial portion of patients with splanchnic venous thrombosis not meeting all the criteria for diagnosis of PV or ET. We have previously reported the presence of the JAK2 V617F mutation in the absence of overt signs of CMD in 21% of overall patients with splanchnic venous thrombosis and in 32% of patients with extrahepatic portal vein thrombosis (De Stefano *et al*., J. Thromb Haemost 5: 708, 2007). Recently additional JAK2 exon 12 mutations have been reported in PV patients JAK2 V617F-negative, two of them with splanchnic venous thrombosis (Scott *et al.,* N Engl J Med 356: 459, 2007; Colaizzo *et al.,* Blood 110:2768, 2007) *Aims.* The present study is aimed to investigate the prevalence of the JAK2 exon 12 mutation. Among patients with splanchnic venous thrombosis and without overt CMD. *Patients and Methods.* We investigated JAK2 exon 12 mutations in 52 patients (M/F 27/25) with splanchnic venous thrombosis and without overt CMD who had been previously tested for the JAK2 V617F mutation and resulted negative. The median age at the thrombotic event was 45 years (range 18-79). Thrombosis involved the extrahepatic portal vein in 31 patients, the superior mesenteric vein in 13, the hepatic veins in 7, and the splenic vein in 1. DNA samples was obtained from peripheral blood granulocytes. We screened the JAK2 exon 12 mutations according to the original reports by Scott et al. (2007) for F537-K539delinsL, H538QK539L, K539L, and N542-E543del, and by Colaizzo *et al.* (2007) for R541-E543delinsK. We tested 46 patients for the F537-K539delinsL mutation, 42 for H538QK539L, 45 for K539L, 40 for N542-E543del, and 50 for R541-E543delinsK. Thirty-six patients were screened for all the 5 mutations. Result. We did not find any mutation of the JAK2 exon 12 in this sample of patients with abdominal thrombosis and JAK2 V617F negative. *Conclusions.* Apparently the JAK2 exon 12 mutations are not frequently detectable in patients with splanchnic venous thrombosis, unlike the JAK2 V617F mutation.

C026

THE COMBINATION OF MEK INHIBITORS AND ARSENIC TRIOXIDE TRIGGERS *IN VITRO* AND *IN VIVO* SYNERGISTIC CYTOTOXICITY IN MULTIPLE MYELOMA THROUGH MULTIPLE SIGNALING PATHWAYS

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We here demonstrate that blockade of the MEK/ERK signaling module, using the small-molecule inhibitors PD0184352 or PD0325901 (PD) (Pfizer), strikingly enhances arsenic trioxide (ATO)-induced cytotoxicity in multiple myeloma (MM) cells through a caspase-dependent mechanism. *In vitro*, the combination PD/ATO shows a moderate inhibitory effect on normal hematopoiesis and has a minimal effect on normal bone marrow B cells. Co-treatment with PD greatly enhances the ATO-induced p53 accumulation and p73, a p53 paralogue, cooperates with p53 in caspase activation and apoptosis induction in MM cells that retain a functional p53 pathway. Co-treatment with PD strikingly elevates the (DR4+DR5)/(DcR1+DcR2) tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors ratio and caspase-8 activation of ATO-treated MM cells that do not have a functional p53 pathway. Bim interacts with DR4 and DR5 TRAIL receptors and contributes to the PD/ATO-mediated activation of the extrinsic pathway. Furthermore, in MM cells carrying or not a functional p53 the combined treatment increases the level of the pro-apoptotic Bim (PD-mediated) and decreases its neutralizing anti-apoptotic protein Mcl-1 (ATO-mediated) and loss of Bim interferes with both the extrinsic and intrinsic apoptotic pathways activation of PD/ATO-treated cells. Studies in a human plasmocitoma xenograft model show that combination of PD (10 mg/kg) and ATO (2,5 mg/kg) is well tolerated, triggers a dramatic reduction (95-98%) in tumor growth relative to untreated mice. Immunohistochemistry and western blot analysis of tumors from treated mice demonstrate that the potent anti-MM activity of PD plus ATO *in vivo* is due to either decreased proliferation (decrement in the number of Ki-67 positive plasma cells and ERK inhibition) and increased apoptosis (Caspase-3 activation). Survival is significantly prolonged in PD/ATO-treated animals versus either treatment alone (mean survival time of PD/ATO 2.5 mg/kg: 74 days; PD: 41 days, p=0.003 for PD/ATO versus PD; ATO 5 mg/kg: 36 days, p=0.001 for PD/ATO versus ATO 5mg/kg; ATO 2.5mg/kg: 27 days, p=0.001 for PD/ATO versus ATO 2.5 mg/kg; untreated: 28 days, p=0.001 for PD/ATO versus untreated). Notably, after 21 days of PD/ATO treatment mice show only moderate bone marrow hypoplasia with respect to untreated animals. These preclinical *in vitro* and *in vivo* studies provide the framework for clinical trials of PD0325901 with ATO to improve patients outcome in MM.

C027

CLINICAL-MOLECULAR PREDICTORS OF THROMBOCYTOPENIA AND RISK OF BLEEDING IN PATIENTS WITH VON WILLEBRAND DISEASE TYPE 2B: A PROSPECTIVE STUDY IN A COHORT OF 67 PATIENTS.

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Background. Type 2B von Willebrand disease (VWD2B) is caused by an abnormal von Willebrand factor (VWF) with increased affinity for the platelet receptor glycoprotein 1b-alpha. This usually results in moderate-severe thrombocytopenia. Aims, Design of the study, Patients and *Methods.* To determine the prevalence and clinical-molecular predictors of thrombocytopenia as well as the risk of bleeding associated with VWD2B we have enrolled 67 VWD2B patients from 38 unrelated families in a 2-year prospective study. At enrolment, patients with phenotypic diagnosis of VWD2B and identified mutation in exon 28 of the VWF gene were also exposed to detailed questionnaire useful to calculate the bleeding severity score (BSS). Platelet counts with mean platelet volume and morphologic evaluations of blood smear were associated with the occurrence of physiological (pregnancy) or pathological (infections, surgeries) stress situations or DDAVP administration. Active VWF was tested in plasma using the AuVWFa11-based immunoadsorbent assay, which allows quantification of the VWF-GpIbIalpha±-binding conformation *in vivo*. Bleeding-free survival according to BSS [<4 (reference), 4-8, >8] and to different platelets levels [≥140×10^9/L (reference), <140×10^9/L] was calculated with the Kaplan-Meier method. *Results.* Thrombocytopenia was found in 20 patients (30%) at baseline and in 38 (57%) after stress situations. Platelet counts were always normal in 16 (24%) patients from 5 families with P1266L/Q or R1308L mutations and normal multimers in plasma. The activated VWF measured by AuVWFa11 nanobody was higher than normal in all but those 16 cases with values 2 to 6-fold higher than controls: values >1 correlated always with thrombocytopenia. Bleeding-free survival calculated with the Kaplan-Meier method was significantly different in the three groups of patients with BSS <4, 4-8 and >8 (log rank test: p=0.003) and in the two groups with platelet counts higher or lower than 140,000//microliters (log rank test: p<0.0001). The adjusted hazard ratio (HR, 95% CI) was about four times higher in patient who had BSS >8 [HR=3.78 (1.00-14.79)] and platelet counts <140,000/microliters [HR=3.65 (1.53-8.70)],

compared to the reference group. *Conclusions.* Not all VWD2B patients show thrombocytopenia at baseline and platelet counts can decrease only after physiological and pathological stress situations. Activated VWF as tested by nanobody is useful to predict thrombocytopenia in VWD2B.

C028

THE HDAC INHIBITOR ITF2357 INHIBITS IL6-RECEPTOR EXPRESSION AND SIGNALING IN MYELOMA AND LEUKEMIA CELLS

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Interleukin-6 (IL6) is a major growth and anti-apoptotic factor in multiple myeloma (MM) and its signaling, mediated by Stat3 activation, has been implicated in growth and metastasis of a range of hematopoietic neoplasms as well as solid tumors. Previously we have shown that ITF2357, a novel hydroxamate histone deacetylase inhibitor, strongly induces apoptosis in MM and AML and inhibits production of growth factors, in particular IL6, by bone marrow stromal cells. In order to determine whether the response to IL6 could also be modulated by ITF2357, we examined the expression of the IL6R alpha and beta chains (CD126 and CD130, respectively) on MM (H929, KMS12, KMS18, U266, CMA03) and AML (KG1, THP1, HL60, NB4, GFD8) cell lines. All the MM and AML lines examined expressed CD126; in contrast 4 of 5 MM lines but none of AML lines express CD130 by FACS analysis. We then analyzed the effect of ITF2357 on IL6R expression. Whereas ITF2357 treatment did not significantly modify CD130 expression, CD126 was strongly down-modulated by the drug in all cell lines: we observed a mean of 85% and 78% decrease in percentage positive cells after 24 hours treatment with 0.5 microM ITF2357 in MM and AML lines, respectively. A down-modulation of CD126 was also observed at the RNA level by quantitative PCR after 6 and 24 hours of treatment with ITF2357.

Table 1.

	Expression CD126 (%)	
	Untreated	*+ ITF2357 24h 0,5 microMolar*
MM - Mean decrease 85 %		
H929	70,3	1,6
KMS12	89,4	9,0
KMS18	18,7	0,8
U266	66,4	11,4
CMA03	64,7	25,3
MM patient	12,3	0,6
AML - Mean decrease 78%		
KG1	81,2	1,9
THP1	98,6	67,4
HL60	29,2	9,9
NB4	34,0	1,0
GFD8	12,6	0,3
AML patient	36,9	0,2

In order to determine whether the decrease in CD126 was accompanied by decreased signaling by IL6R, we measured Stat3 phosphorylation in presence and absence of IL6 and/or ITF2357. As expected, MM cells responded to exogenous IL6 with an intensification of Stat3 phosphorylation. This signaling was decreased by the drug treatment at least to the basal level, also in cell lines that were relatively resistant to apoptosis induction by ITF2357 (U266 and GFD8). Interestingly, the AML cell lines which appeared to express only CD126 also responded to IL6 by Stat3 phosphorylation and this response was down-modulated by ITF2357, presumably due to low level of CD130 expression. We also analyzed freshly isolated MM and AML cells and found that ITF2357 down-modulated CD126 in these cells and inhibited signaling by IL6. To conclude, these data suggest that the new iHDAC ITF2357 has a dual activity versus IL6 signaling: on the one hand it inhibits IL6 production by stromal cells and on the other it blocks the activity of this cytokine on myeloma and leukemic blasts, by down-modulating IL6R expression.

C029

ZOLEDRONIC ACID INCREASES THE *IN VITRO* APOPTOTIC EFFECT OF BORTEZOMIB AND MAY OVERCOME DRUG RESISTANCE IN HUMAN MYELOMA CELLS

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Myeloma cells are extremely resistant to drug-induced apoptosis and multiple myeloma (MM) remains an incurable disease. In the last years novel agents have been introduced in the therapy of MM as Bortezomib and thalidomide however new therapies or novel drug combinations need urgently. Growing evidences suggest that zoledronic acid (ZOL) may have anti tumoral effects in MM including a direct *in vitro* pro-apoptotic on MM cells. A synergistic effect has been also observed between ZOL and dexamethasone or thalidomide in MM cells. In this study we have investigated the potential *in vitro* effect of ZOL in combination with Bortezomib and the capacity of ZOL to overcome drug resistance in human MM cell.First the effect of ZOL was checked at concentration ranging between 10-4 M to 10-6 M both in fresh purified $CD138^+$ MM cells isolated from patients at the diagnosis or relapse and in the human myeloma cell line JJN3 in combination with Bortezomib at concentration ranging between 2 nM and 10 nM. We found that the combination of ZOL (10-5M) with Bortezomib significantly increased the inhibitory effect on MM cell proliferation by Bortezomib. Similarly we found that ZOL (10-4 M) significantly increased the apoptotic effect of Bortezomib inducing caspase-8 activation and the down-regulation of the pivotal anti apoptotic molecule Mcl-1. In addition, we found that ZOL induced apoptosis of the Bortezomib resistant cells DHL-4 and RPMI-R5 and it overcome Bortezomib resistance in these cell lines. This effect was further analyzed evaluating the potential effect on gene expression profiling by microarray (Affymetrix) identifying genes differently modulated by ZOL and by the combination of ZOL plus Bortezomib in RPMI-R5. The capacity of ZOL to increase drug-induced MM cell apoptosis was also observed with other potential anti MM drugs as arsenic trioxide (AS_2O_3). We found that the combination of ZOL with AS_2O_3 significantly increased the inhibitory effect on MM cell proliferation by AS_2O_3 alone and it induced a significant anti-proliferative effect on MM cell resistant to AS_2O_3. Similarly we found that ZOL significantly increased the apoptotic effect of AS_2O_3.In conclusion our data indicate that ZOL at high concentrations increases the apoptotic effect of Bortezomib and AS_2O_3 and may overcome drug-resistance in MM cells.

C030

PROCOAGULANT ACTIVITIES OF NB4 ACUTE PROMYELOCYTIC LEUKEMIA CELLS ARE INHIBITED BY ARSENIC TRIOXIDE: COMPARISON WITH ALL-TRANS RETINOIC ACID

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ATRA and ATO, as single agents, induce the molecular remission of APL and induce a simultaneous rapid resolution of the related coagulopathy. Furthermore the combination therapy of ATRA+ATO is effective in inducing APL remission in newly diagnosed patients, and may provide an alternative to ATRA+chemotherapy in this disease. One mechanism of ATRA effect on the coagulopathy relies on ATRA capacity to reduce APL cell procoagulant activities (PCA) [i.e. tissue factor (TF) and cancer procoagulant (CP)]. However, little information is available on the effects of ATO or the combination ATO+ATRA on the PCA of APL cells. Aim of this study was to evaluate the capability of ATO and ATO+ATRA, as compared to ATRA alone, to modulate TF and CP of the NB4 APL cell line. The association between the PCA variations and the occurrence of cell differentiation, proliferation, and apoptotic/necrotic phenomena was also explored. NB4 cells were incubated with either 0.1 μM ATO, 1 μM ATRA, 0.1 μM ATO/1 μM ATRA or the vehicle (control cells). At different incubation times (i.e. 24, 48, 72 and 96 h) cells were tested for TF and CP expression, cell differentiation (as an increase in CD11b expression), proliferation and apoptosis/necrosis (by annexin V/propidium iodide staining). The results show that, starting from 24 h incubation, TF activity was 41% reduced by ATO ($p<0.05$) and 89% by ATRA ($p<0.01$). The reduction in TF activity was associated with a parallel and similar decrease in TF mRNA and TF antigen levels. CP activity was 18% reduced by ATO ($p<0.05$) and 61% by ATRA ($p<0.01$). The ATO+ATRA combination was more effective in modulating the two procoagulants

than ATO, with no significant difference from ATRA alone. Simultaneously to the PCA downregulation, cell proliferation was inhibited by ATO by inducing cell necrosis, and by ATRA or ATO+ATRA by inducing apoptosis and cell differentiation, without necrosis. These data indicate that ATO can modulate both TF and CP activities of NB4 cells, although less than ATRA, and, in combination with ATRA, its effect on PCA is augmented. It is important that, differently from ATO alone, the ATRA+ATO combination is not associated to the induction of necrotic cell death which often causes extensive tissue damage and intense inflammatory response. A role for ATO in the molecular-targeted anticoagulant control of APL-associated coagulopathy is suggested.

C031

GENE EXPRESSION PROFILING OF MULTIPLE MYELOMA: IMPLICATIONS FOR A MOLECULAR CLASSIFICATION OF THE DISEASE

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Multiple myeloma (MM) is a neoplastic disorder of bone marrow plasma cells (PCs), which can be preceded by a monoclonal gammopathy of undetermined significance (MGUS) and may progress to plasma cell leukemia (PCL). We analyzed the gene expression profiles of highly purified PCs isolated from 12 MGUS, 132 MM and 9 PCL patients and 4 healthy donors (N) using HG-U133A arrays. The entire dataset, described by 597 highly variable probes, generated a dendrogram with two major branches, one containing 10/11 MGUS and N cases, whereas the other clustering all the PCLs and most of the MMs. Neither PCL nor MM samples could be identified as a distinct cluster. A multi-class analysis revealed 287 probe sets specifically distinguishing MGUS from PCL samples and showing a progressive modulation in MM cases, during the transition from MGUS to the PCL phenotype. The up-regulation of immune response genes was evidenced in MGUS cases, whereas primary metabolism, cell cycle and apoptosis induction processes were positively modulated in PCLs. Clustering of the 552 highly variable probes in 132 MM database was mainly driven by groups defined according to TC classification (based on the presence of IGH translocation and Cyclins D expression). One main branch contained 23/24 (p=10^{-4}) TC2, 18/29 TC1/t(11;14) and approximately half (13/24) TC4/t(4;14) samples, while the other major branch grouped the remaining TC1 and TC4 cases together with a sub-cluster of all (6/6, p=2.1×10^{-3}) TC5 (MAF) samples. The 49 TC3 cases were spread all over the clustering, in accordance with the absence of differentially expressed genes in a multi-class analysis. A contingency study showed an extremely significant correlation between 13q deletion and TC4 class (19/21, p=10^{-4}) and between its absence and the TC2 group (19/21, p=10^{-4}); hyperdiploid status was strongly associated to TC2 and TC3 classes (38/53, p=10^{-4}), while 1q/gain was significantly absent in TC2 group (16/19, p=8×10^{-4}) and present in TC3 (26/39, p=7.8×10^{-3}); 17p deletion was only related to TC1 cases (5/24, p=3.7×10^{-2}), whereas the presence of focal bone lesions was strictly correlated to CCND1 over-expressing groups (TC1 and TC2; 30/41, p=$4.4\times10\times10^{3}$). In general, our study supports the notion of a marked heterogeneity of MM and highlights the association of distinct gene expression patterns with specific genetic lesions, providing important contributions to a molecular classification of the disease.

C032

BONE MICROENVIRONMENT CELLS REVEALS A DIFFERENT GENE EXPRESSION PROFYLING IN MULTIPLE MYELOMA PATIENTS IN RELATION TO THE BONE STATUS

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Multiple myeloma (MM) is characterized by the high capacity to induce osteolytic bone lesions. Osteoblasts (OB) formation is impaired in MM patients mainly due to the suppression of the osteogenic differentiation of human mesenchymal cells (MSC)/osteoprogenitor cells. MSC and OB cells isolated from bone biopsies were profiled by means of HG-U133A arrays (Affymetrix), to find the gene expression alterations in the bone microenvironment cells and their potential relationships with bone lesions occurrence in MM patients. An unsupervised analysis of the most variable genes across the entire database generated a hierarchical clustering with two major branches distinguishing MSC and OB cellular types. A preferential clustering in relation to the presence of osteolytic bone lesions was observed only for MSC not for OB samples, when considering each dataset. Furthermore, the positive modulation of 134 genes and the down-regulation of 45 genes were observed comparing the 8 osteolytic versus the 8 non-osteolytic MSCs. Nucleic acid metabolism (37%), particularly nuclear mRNA splicing (10%), intracellular transport (10%) and cell-cycle (4%) genes resulted positively modulated, while inflammatory response (9%), Wnt-signalling pathway (FRAT1, WNT16, WNT6) genes and extracellular matrix structural constituents (COL3A1, DCN, PRELP) were found down-regulated in osteolytic MSCs. No significant altered genes were found between OB samples in relation to bone lesions occurence. Additionally, 119 and 246 genes were found as specifically modulated in the non-osteolytic (8 MSC versus 10 OB) and in the osteolytic (8 MSC versus 6 OB) groups, respectively, showing a greater gene expression variation among the two cellular types in presence of bone lesions. Protein modification, proteolysis and transport, cell cycle arrest and pro-apoptotic response were found as positively regulated processes in non-osteolytic OBs with respect to MSCs, whereas the up-regulation of amino acid biosynthesis and translation and the down-regulation of M phase and cytoskeleton biogenesis/organization genes were evidenced in osteolytic OBs. Commonly negatively-modulated functions in OBs compared to MSCs were response to DNA-damage stimulus, processing of mRNA and generation of precursor metabolities and energy. Our results highlight the presence of genes alterations in bone microenvironment cells potentially involved in the development of bone lesions in MM patients.

Chronic Lymphocytic Leukemia and Lymphomas

C033

IDENTIFICATION BY COMPARATIVE GENOME-WIDE DNA PROFILING OF RECURRENT GENETIC LESIONS SPECIFICALLY ASSOCIATED WITH HIV-RELATED NON-HODGKIN LYMPHOMA

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Non-Hodgkin's lymphomas (NHL) represent a frequent complication of HIV infection and a major source of morbidity and mortality among patients affected by acquired immunodeficiency syndrome. Data on the underlying genetics of HIV-NHL are still scarce and mainly based on screening of genes known to be involved in the pathogenesis of lymphoma in immunocompetent (IC) hosts. The aim of this study was to improve our understanding of HIV-NHL pathogenesis using a powerful analysis technique, such as genome-wide DNA profiling. SNP-microarray comparative genomic hybridization was performed in 70 HIV-NHL, including 59 systemic cases (29 HIV-diffuse large B-cell lymphomas, HIV-DLBCL; 22 HIV-Burkitt lymphomas, HIV-BL; 8 HIV-Burkitt-like lymphomas, HIV-BLL), as well as 4 HIV-primary central nervous system lymphoma (HIV-PCNSL) and 7 HIV-primary effusion lymphomas (HIV-PEL). Twenty-two IC-DLBCL were analyzed as a control group. DNA samples, extracted from frozen biopsies, were analyzed using the Affymetrix Human Mapping 250K Nsp arrays. Methylation analysis of selected genes was performed by methylation specific PCR. Aberrations occurring in 20% or more of the cases were defined as recurrent. HIV-DLBCL cases had recurrent gains at 2p15, 3q29, 4q13.2, 8p23.1, 10p12.31, 11q22.1-q24.1, 14q11.2, 14q32.33, 12p13.31-pter, 15q11.2, and recurrent losses at 1p36.32-p36.33, 2p11.2, 5p12, 3p14.2, 7q22.1, 8p23.1, 9p23, 9p24.1 12p11.1, 14q11.2, 15q11.2, 17p13.1-p13.3 and 22q11.1. In comparison to IC-DLBCL, significant differences were the lack of 18q gains, and a lower frequency of 6q losses. HIV-BL had recurrent gains in 1q25.2, 3q29, 8p23.1, 15q11.2, 12p12.1-pter, 20q13.12 and recurrent losses at 15q11.2 and 17p11.2-p13.2. Frequent alterations of specific genes were also observed. Deletion of the fragile histidine triad gene (FHIT), in the recurrently lost 3p14 region, was detected in 14/70 (20%) HIV-NHL (6 HIV-DLBCL, 4 HIV-PEL, 2 HIV-BL and 2 HIV-BLL). Deletion of the WW domain-containing oxidoreductase gene (WWOX), at 16q23.1, was observed in 9/70 (13%) cases (4 HIV-DLBCL, 4 HIV-PEL and 1 HIV-BL). In all but one cases, WWOX deletion was associated with FHIT deletion. No IC-DLBCL had interstitial deletions affecting either FHIT or WWOX. Methylation of the FHIT gene was observed in 14/70 (20%) HIV-NHL (6 HIV-DLBCL, 6 HIV-BL, 1 HIV-BLL and 1 HIV-PCNSL), whereas WWOX methylation was observed in 31/70 (44%) cases (15 HIV-DLBCL, 7 HIV-BL, 3 HIV-BLL, 2 HIV-PCNSL and 4 HIV-PEL). Reduced mRNA expression of WWOX and FHIT was demonstrated, by quantitative real time PCR, in cases with gene methylation and/or deletion. Our data show evidence of specific genomic alterations associated to HIV-NHL. FHIT and WWOX are selectively inactivated by DNA loss and/or promoter methylation in about 50% of HIV-NHL. HIV-BL shows less copy number aberrations than HIV-DLBCL. The pattern of gains and losses in HIV-DLBCL is more similar to the genetic profile observed in IC DLBCL derived from germinal center B-cells (GCB) than that of DLBCL derived from activated B-cells (ABC).

C034

CD38 AND ZAP-70 ARE INVOLVED IN CXCL12-MEDIATED CHEMOTAXIS OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Chronic lymphocytic leukemia (CLL), traditionally regarded as a disease of failed apoptosis, is now considered as a disease characterized by different propensity to grow according to microenvironmental signals. Absence of mutations in the IgV genes, expression of the surface receptor CD38 and of the cytoplasmic kinase ZAP-70 are acknowledged independent markers of unfavorable outcome in CLL patients. Our group investigates the role of CD38 in CLL not only as disease marker but also as pathogenetic agent. CLL cells recirculate from blood to peripheral lymphoid organs, where a favorable microenvironment provides growth and survival signals mediated - at least in part - by CD38. We showed that CD38 enhances proliferation/survival of CLL cells through binding its non soluble ligand CD31, expressed by stromal, endothelial and nurse-like cells (NLC). Further, i) CD38 engagement leads to tyrosine phosphorylation of ZAP-70, ii) CD38 pathway is impaired in $CD38^{+}/ZAP\text{-}70^{-}$ cells and iii) $CD38^{+}/ZAP\text{-}70^{+}$ cells efficiently migrate towards CXCL12, abundantly produced by NLC. Collectively, these data point to a functional role for CD38 and ZAP-70 in CLL migration towards this chemokine. Indeed, CXCL12-mediated chemotaxis can be blocked by anti-CD38 monoclonal antibodies (mAbs). Pre-treatment of CLL cells with anti-CD38 mAbs impairs CXCR4 signaling, as indicated by lack of Ca^{2+} mobilization, ERK1/2 activation and receptor internalization. Further, CD38 and CXCR4 are physically associated, as shown by confocal microscopy and co-immunoprecipitation. The above results obtained *in vitro* were then confirmed in an *in vivo* model: anti-CD38 mAbs efficiently decrease homing of CLL cells injected into the tail vein of NOD/SCID mice.In conclusion, these results offer biological evidence explaining why the combination of CD38 and ZAP-70 in clinical routine results in a more efficient identification of CLL patients with aggressive disease. Further, they represent preliminary rationale for using anti-CD38 ligands or inhibitors as adjunct therapeutic tools.

C035

GENOME-WIDE DNA ANALYSIS IDENTIFIES RECURRENT IMBALANCES PREDICTING OUTCOME IN CHRONIC LYMPHOCYTIC LEUKEMIA WITH 17P DELETION

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Deletion of 17p(p53) identifies an infrequent subset of chronic lymphocytic leukemia (17p- CLL) with aggressive behavior. Genome-wide DNA analysis of copy number (CN) changes and loss of heterozygosity (LOH) has provided a tool to identify common recurrent chromosomal changes of pathogenetic significance in cancer. Chromosomal imbalances within 17p- CLL subset have not yet been defined. Genome-wide DNA-profiling using the GeneChip Human Mapping 250K-NspI arrays (Affymetrix, Santa Clara, CA, USA) was performed to identify the genotype of 18 patients with 17p- CLL. The 17p-CLL investigated had significantly shorter time to first treatment (TTT, 16 months, p=0.00004) and overall survival (OS, 104 months, p=0.0000001) than a control cohort of 106 non17p- CLL (TTT 65 months and OS not reached, respectively). All samples carried 17p13.1 deletion in more than 60% nuclei, as required for efficient genome profiling. Genome-wide DNA profiles had a concordance rate with FISH results for 11q23, 12p11-q11, 13q14.3 and 17p13 of >98% (71/72 datapoints). The vast majority (15/18) of 17p(TP53) deletions targeted the whole 17p arm, while the smallest deletion was 1 Mb long. Deletions of 13q14.3 locus occurred in 9/18 (50%) cases, trisomy 12 in 1/18, while none had 11q23 (ATM) deletion. The 17p

deletion was never the sole aberration and all cases had multiple DNA imbalances. The remaining most common deletions affected several loci on 8p (8p12, 8p21.1-p23.1, 8p23.1-p23.3), and the 9q21.33-q22.2 region. The most frequent gains targeted chromosome arms 2p (2p14-p16.1, 2p22.3-pter), 3q (3q24-q29), 8q (8q23.3-qter, 8q24.13-q24.1 that contains cMYC, 8q24.3), and 17q (17q21.2-q21.32, 17q21.32-q22). Among these CN changes, 8p-loss (TRAIL-R1/2) and 2p16.1-p14-gain (REL/BCL11a) appeared clinically relevant and predicted significant shorter time from diagnosis to treatment (8p-loss, p=0.04) and overall survival (8p-loss, p=0.04 and 2p16.1-p14-gain, p=0.02). A high frequency of loss of heterozigosity (LOH), with 6 patients displaying copy-neutral LOH regions, was also demonstrated. In conclusion, our analysis revealed a high frequency of recurrent genomic aberrations, which may identify mechanisms of poor behaviour and contribute to the bad prognosis of 17p- CLL. The remarkable significant association of 8p-loss and 2p14 gain with prognosis even in this small cohort claims for the role of specific genes other than TP53 in controlling tumour behaviour and activity within the 17p- CLL.

C036

GENERATION OF A NEW CHIMERIC ANTIGEN RECEPTOR TO TARGET CD23 EXPRESSED ON CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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B-Chronic lymphocytic leukemia (B-CLL) is characterized by progressive accumulation of mature B-lymphocytes expressing CD19, CD20dim and aberrantly expressing the CD5 T-cell marker. Moreover, they over-express the B-cell activation marker CD23. Chimeric Antigen Receptors (CAR) are engineered molecules constituted by an antigen-recognizing antibody chain linked to a T-cell triggering domain. CARs are able to redirect T-cell specificity and their killing/effector activity towards a selected target in a non MHC-restricted manner. First trials targeting CLL were based on both monoclonal antibodies and anti-CD19/anti-CD20 CAR-transduced T cells. However a pitfall of this approach is the elimination of normal B-lymphocytes with consequent impairment of humoral responses. Selective CD23 expression on CLL cells renders CD23 an optimal target to design a specific CAR. We cloned a new CD23-targeting CAR to redirect T cells against $CD23^+$ B-CLL. Preliminary results indicated that anti-CD23 CAR is expressed on T cells (with an average level of transduction of 20%; range,10%-60%, n=10) and confers them a strong cytotoxicity against CD23+ tumor cell lines (Epstein Barr Virus transformed lymphoblastoid cell line, average lysis 50%, range 15%-70%, at Effector:Target Ratio (E:T) =40:1, n=5; Bjab and Jeko cell lines transduced with human CD23 antigen, average lysis 60%, range 20-75%, at E:T= 40:1, n=3;). On the contrary, anti-CD23 transduced T-cells displayed no relevant killing activity versus normal B cells (with an average level of 8%, range 1%-15% at E:T ratio 40:1), differently from anti-CD19 CAR redirected T-cells, which kill tumor and normal B cells in an indistinct manner. Further experiments are necessary to optimize CAR functionality and test its efficacy in an autologous contest, as well its safety profile towards normal B lymphocytes.

C037

THE PHARMACOGENETIC BACKGROUND OF THE HOST IS AN INDEPENDENT PREDICTOR OF OUTCOME AND TOXICITY IN DIFFUSE LARGE B-CELL LYMPHOMA TREATED WITH R-CHOP21

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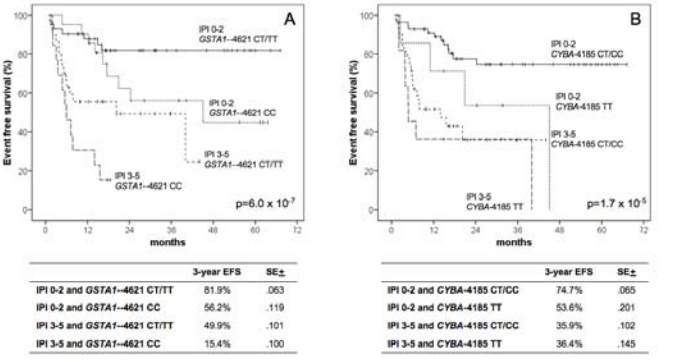
R-CHOP is the standard treatment for diffuse large B-cell lymphoma (DLBCL). Several tumor cell-related predictors of efficacy and toxicity have been so far identified for DLBCL treated with R-CHOP. Conversely, no information is available on the impact of the host's genetic background as a predictor of outcome and toxicity in this context. This study aimed at verifying whether relevant single nucleotide polymorphisms (SNPs) of the host may further refine the prognostic stratification and the prediction of toxicity in DLBCL patients treated with R-CHOP21. The study was based on a consecutive series of 106 DLBCL treated with R-CHOP21 and provided with a large and homogeneous dataset comprising clinical variables at diagnosis, chemotherapy doses and toxicity. All data were collected prospectively. Candidate SNPs belonged to genes known to be involved in the pharmacogenetics of cyclophosphamide, doxorubicin, vincristine, prednisone and rituximab, and included SNPs affecting: i) drug metabolism (cytochrome-P450: CYP2B6-25504C>T, CYP2B6-15630G>T, CYP2C19-19153G>A, CYP3A4--392A>G, CYP3A4-14365T>G, CYP3A5-6980G>A); ii) drug detoxification: (gluthatione-S-transferase: GSTA1--4621T>C, GSTM1-null, GSTP1-1374A>G, GSTP1-2264C>T); iii) drug transport (multidrug-resistance-related proteins: ABCC1-129623G>T, ABCC2-53394T>A, ABCC2-68692G>A, ABCB1-90855C>T, ABCB1-49691G>A, ABCG2-8824C>A, ABCG2-33G>A; and iv) drug pharmacodynamics (NADPH-subunits: NCF4--368A>G, RAC2-7418T>A, CYBA-4185C>T; TP53: p53-440G>C; Fc receptor: FCGR2A-4487A>G, FCGR3A-5092T>G, FCGR3A-1301T>G/A; Glucocorticoid receptor: GR-1087A>G, GR-67G>A, GR-1829C>G). Genotyping of candidate SNPs was performed on PBMC collected at DLBCL diagnosis by SNP-minisequencing (ABI Prism SNaPshot Multiplex kit, Applied Biosystem). Primary end points were event free survival (EFS) and toxicity. Univariate log-rank analysis identified CYBA-4185C>T (3-year EFS CT/CC: 61.2% *vs.* TT 41.7%; p=.008), GSTA1--4621C>T (3-year EFS CT/TT: 64.8% *vs.* CC: 39.2%; p=.027), and ABCC2-53394T>A (3-year EFS TT: 64.0% *vs.* AT/AA: 34.1%; p=.013) as predictors of EFS. Multivariate analysis identified CYBA-4185TT (HR:2.57; p=.010) and GSTA1--4621CT/TT (HR:0.44; p=.010), along with chemotherapy interruption (HR: 3.52; p=.002), IPI3-5 (HR:2.81; p=.006), and liver involvement (HR:3.07; p=.006) as independent predictors of EFS after adjusting for ABCC2-53394T>A, marrow involvement, organ function (marrow, renal, liver, cardiac), comorbidities, and dose intensity of cyclophosphamide and doxorubicin. CYBA-4185C>T and GSTA1--4621C>T also predicted outcome in DLBCL subgroups by IPI (Figure 1). The impact of SNPs was also evaluated for toxicity in 658 courses of R-CHOP21 by logistic regression analysis adjusted for age, sex, ECOG PS, IPI, comorbidities, organ function, and doses of cyclophosphamide, doxorubicin and vincristine. NCF4--368AG/GG was an independent predictor of low risk of hematologic toxicity G3-4 (HR:0.49; p=.012), infection (HR:0.42; p=.002), cardiac toxicity (HR:0.34; p=.001), and neurological toxicity (HR:0.52; p=.009). The implications of these results are twofold. First, host SNPs affecting doxorubicin and/or cyclophosphamide pharmacodynamics (CYBA-4185C>T) and detoxification (GSTA1--4621C>T) are independent predictors of outcome in DLBCL treated with R-CHOP21. Poor EFS heralded by CYBA-4185C>T, a nonsynonimous SNP of NADPH-oxidase p22phox, may be due to reduced production of reactive oxygen species (ROS) that mediate doxorubicin cytotoxicity. Favorable EFS associated with GSTA1--4621C>T may be related to reduced expression of glutathione-S-transferase A1, a phase II enzyme involved in cyclophosphamide and doxorubicin detoxification. Second, NCF4--368AG/GG, a SNP belonging to NADPH-oxidase p40phox and regulating ROS generation, has an independent protective role against both hematologic and non-hematologic toxicity.



	3-year EFS	SE±
IPI 0-2 and *GSTA1--4621 CT/TT*	81.9%	.063
IPI 0-2 and *GSTA1--4621 CC*	56.2%	.119
IPI 3-5 and *GSTA1--4621 CT/TT*	49.9%	.101
IPI 3-5 and *GSTA1--4621 CC*	15.4%	.100

	3-year EFS	SE±
IPI 0-2 and *CYBA-4185 CT/CC*	74.7%	.065
IPI 0-2 and *CYBA-4185 TT*	53.6%	.201
IPI 3-5 and *CYBA-4185 CT/CC*	35.9%	.102
IPI 3-5 and *CYBA-4185 TT*	36.4%	.145

Figure 1.

C038

CLINICAL AND BIOLOGICAL CHARACTERIZATION OF CIRCULATING ENDOTHELIAL CELLS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Several studies have shown that bone marrow-derived endothelial cells (EC) may contribute to tumor angiogenesis and that in the peripheral blood of cancer patients there is an increased amount of circulating ECs (CECs) that may participate to new vessel formation. Recent data also showed that in B-cell neoplasms ECs are in part tumor-related reflecting a novel aspect of tumor angiogenesis. All together these observations suggest that tumors can elicit the sprouting of new vessels from existing capillaries through the secretion of angiogenic factors and that, in some cases, cancer cells can also mimic the activities of ECs by participating in the formation of vascular-like networks. In this study we aim to characterize the clinical and biological role of CECs in a series of 85 chronic lymphocytic leukemia (CLL) patients. CEC levels were evaluated by multiparameter flow cytometry and correlated with known clinical and biological parameters. For biological studies, CECs were first isolated by immunomagnetic sorting and then characterized by phenotypic studies with antibodies recognizing endothelial and CLL antigens, by FISH analyses with specific probes and by gene expression profiling comparing CLL CECs with CECs from normal subjects, and with monocytes and lymphocytes from the same CLL patient. We found that CEC levels were significantly higher in CLL patients in comparison to normal healthy subjects (p=0.037). Higher CEC levels were associated with advanced disease stage (p=0.012) and with lack of response to treatment or progressive disease (p=0.005). No association was demonstrated with CD38/ZAP70 expression and FISH/cytogenetic abnormalities. In all experiments more than 95% of immunomagnetically sorted cells were of EC origin as demonstrated by phenotypic analyses (VEGFR2$^+$, vWF$^+$, CD144$^+$, UEA1 lectin$^+$, CD45$^-$, CD14$^-$, CD5$^-$, CD19$^-$). FISH analysis showed that a significant proportion of sorted CECs was tumor-derived because they harbored the same genetic lesion as observed in neoplastic CLL cells. The fraction of CECs showing cytogenetic aberrations averaged 40.7% (range, 20-78%). More then 85% of CECs presented features of EPCs because they expressed CD133, a marker gradually lost during EC differentiation and absent in mature ECs. CLL CEC had a similar gene expression pattern for several genes characterizing CEC function such as CD144, CD34, CD133, CD146, CD31, VEGFR2, VEGFR3, VWF, and TIE2. Moreover, CLLCEC showed a strongly different gene expression pattern compared to normal CEC characterised by increased cell survival and proliferation including activation of Wnt and inhibition of Notch signalling pathways, reduction of cell adhesion to extracellular matrix and enhanced pro-angiogenic function. Gene expression profiling analysis also suggested that similarities exist with clonal CLL lymphocytes. In conclusion these findings suggest that in CLL CECs are in part tumor related and with a gene expression profile that may indicate their contribution to tumor neovasculogenesis and possibly to the spreading and progression of the disease.

C039

P53 EVALUATION IN 98 CLL PATIENTS BY AMPLICHIP P53 ARRAY REVEALS A HIGH PERCENTAGE OF MUTATIONS AND POLYMORPHISMS

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Background. In chronic lymphocytic leukemia (CLL), inactivation of the TP53 gene is an important poor-outcome marker. In order to characterize a large cohort of CLL cases for TP53 mutations, the Amplichip p53 array – designed by Roche Molecular Systems and manufactured by Affymetrix – was used. Patients and Methods. We evaluated 98 untreated CLL patients, including 59 cases with progressive disease, 28 with stable disease and 4 with stable disease at the time of the study, who later evolved to progressive disease; finally, 7 had a follow-up <40 months. Concordance between IgVH status and ZAP70 expression was 74%, while concordance with CD38 was 79%. All cases were also screened by TP53 sequencing and FISH analysis. The Amplichip p53 array allows sequencing the entire coding region including exons 2-11 splice sites. Genomic DNA is amplified in two PCR reactions; PCR products are then fragmented, hybridized, stained and scanned. The whole procedure requires 8 hours. Each array queries 1250 positions, investigated by multiple probesets tiled for each base positions. Each probeset contains five probes: 1 for wild-type sequence, 3 for possible single base pair substitutions and 1 for single base pair deletion. The mutation detection algorithm detects single base pair substitutions and deletions. Results. The AmpliChip p53 array detected 15 mutations in 13 patients, with an incidence of 19% and 4% in progressive and stable cases, respectively. Remarkably, TP53 sequencing failed to detect 6 mutations (2 at exon 5, 2 at exon 7, 1 microdeletion at exon 6 and 1 mutation at splice sites of intron 3), showing a higher sensitivity of the p53 array; consistently, all mutations identified by p53 sequencing were detected by the p53 array. The AmpliChip p53 array also identified polymorphisms at codon 36, 72 and 213 - at exon 4 and 6 - in 5%, 39.8% and 8% of CLL cases, respectively. Finally, correlation between TP53 mutations and different biologic parameters, i.e. IgVH status, ZAP70 and CD38 expression, status of disease and del17p by FISH, highlighted a significant association only with del17p (Fisher exact test = 0.0048). Conclusions. The p53 array is a simple, sensitive and specific method for TP53 mutational analysis. This approach reveals a high percentage of TP53 mutations in CLL with unfavorable outcome and a significant association between TP53 aberration and del17p. Further investigation on the role of codon 72 polymorphisms, highly represented in CLL, is ongoing.

C040

VARIABLE VASCULARIZATION IN BONE MARROW COMPARTMENT IS PRESENT IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS: ASSOCIATION WITH EXPRESSION OF PRO-ANGIOGENIC FACTORS AND PROGNOSTIC PARAMETERS

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Increasing evidence shows that hematological malignancies, as B-cell chronic lymphocytic leukemia (CLL), may benefit of additional vessels in infiltrated bone marrow (BM). We detected the degree of BM angiogenesis in 26 CLL patients by immunohistochemistry using antibodies to CD34. Heterogeneous BM angiogenesis was observed among patients (range,14.3-39.4): low vascularization levels (<20 microvessel density for high power field, MVD/hpf) were observed in 8 CLL (30.8%), whereas 6 cases (23.1%) showed highly vascularized BM tissue with 30 MVD/hpf and higher. We found that vascularization levels were not correlated with percentage of BM infiltration by CLL cells (p=0.132). Moreover, we detected a trend for BM angiogenesis to increase in CLL with advanced Binet stages (median, 19.7, 27.1 and 29.8 in stages A, B and C). The majority (14/17, 82.4%) of CLL patients with advanced Binet stages (B,C) had increased microvessel count (>20 MVD/hpf). Variable levels (4-fold change) of BM angiogenesis among Binet stage A patients were observed. All three stage A CLL with higher MVD/hpf (32.3, 38.3 and 39.4) belonged to Ig-unmutated (UM-) subset. Interestingly, we found that microvessel density was significantly higher in UM- than in Ig-mutated (M-) CLL subset (28.5 vs. 19.4, p=0.012). Comparing BM angiogenesis with gene expression of pro-angiogenic factors Ang2, bFGF and VEGF, we observed enhanced microvessel density in CLL with increased Ang2 expression. Dividing CLL into low MVD and high MVD groups (cut-off equal to 25 MVD/hpf), we did not find different expression of VEGF and bFGF in both groups (median VEGF, 0.08 and 0.09, p=0.833; bFGF, 1.58 and 2.79, p=0.598), whereas increased Ang2 expression was present in high MVD CLL subset (median, 0.42 and 14.8 in low and high MVD CLL; p=0.009). Likewise, we observed moderate positive correlation between the quantity of Ang2 mRNA and the extent of vascularization in CLL-infiltrated BM microenvironment (r=0.40, p=0.044, Pearson correlation). In conclusion, heterogeneous levels of BM angiogenesis were observed among CLL patients. Of interest, we first reported elevated levels of BM microvessel density in patients harbouring unmutated immunoglobulin genes compared to mutated ones, implying that increased angiogenesis may play a role in different clinical aggressiveness of these CLL subsets. In addition, our results suggest the involvement of Ang2 in CLL angiogenic capacity.

Acute Lymphoblastic Leukemia

C041

UNIQUE GENOMIC PROFILE OF INFANT ALL PATIENTS

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In childhood ALL, individual genetic lesions alone are insufficient to generate a full leukemic phenotype, and cooperating oncogenic lesions are required. Recently, genome-wide studies on childhood (1-18 years) and adult ALL identified deletions at several loci, affecting genes with a critical role in B-cell development. By contrast, the pathogenesis of Infant ALL (<1 year at diagnosis) is not defined. Infant ALL is a very aggressive disease, with t(4;11)/MLL-AF4 fusion as the major subgroup. Although the very short latency suggests that leukemogenic events occur prenatally, mice models indicate that MLL-AF4 alone is not sufficient to induce leukemia, and additional mutations may occur. Aim of this study was to detect MLL-cooperating aberrations, undetectable by conventional techniques. We applied genome-wide SNParray analysis (100K SNP, Affymetrix) to search for Loss of Heterozygosity (LOH) associated or not to copy number alterations. The identification of these lesions could help identifying leukemia pathogenesis, providing also the basis for targeted therapy. We have analyzed 28 cases of Infant ALL with t(4;11) at diagnosis, remission and relapses. Compared to older ALL patients, a far limited number of deletions/amplifications has been found (6 deletions only in 4/28 patients). By contrast, several segmental copy-number neutral (CNN) LOH have been detected and validated by direct sequencing. Genes mapped in recurrent CNN-LOH regions are involved in pathways frequently altered in human cancer, the most prevalent being the DNA repair pathway (FANC genes, BRCA1, RAD family genes). In most cases the same homozygous traits found at diagnosis were also present at remissions, indicating constitutional events. The functional role of CNN-LOH still needs to be understood: they could either reflect the duplication of oncogenic mutations, or be related to epigenetic mechanisms. Overall, these results indicate that, differently from all older ALL cases, MLL rearrangement in Infant ALL is not associated to further deletions. More likely, it can be argued that MLL rearrangement as a first hit, in a genomic background with homozygous stretches affecting genes of fundamental cellular pathways, could have a major role in driving and speed up the leukemogenesis. Alternatively, the constitutional nature of CNN-LOH could indicate a genomic predisposition to the development of the disease, favouring the occurrence of MLL rearrangement itself.

C042

IKZF1 (IKAROS) DELETIONS ARE A CENTRAL EVENT IN THE PATHOGENESIS OF BCR-ABL1 POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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The Philadelphia chromosome is the single most significant adverse prognostic marker in adult ALL. Expression of BCR-ABL1 in haematopoietic stem cells can alone induce a CML-like myeloproliferative disease, but cooperating oncogenic lesions are required for the generation of a blastic leukemia. To identify, at submicroscopic level, oncogenic lesions that cooperate with BCR-ABL1 to induce ALL we profiled the genomes of 36 Ph$^+$ adult ALL patients by 250K NspI single nucleotide polymorphism (SNP) array (Affymetrix Inc., USA). High level amplification and homozygous deletion were identified in all patients, with deletions outnumbering amplification almost 3:1. The most frequent somatic copy number alteration was deletion of IKZF1, which encodes the transcription factor Ikaros, in 25/36 cases (69%). Ikaros is required for the earliest stages of lymphoid lineage commitment and acts as tumor suppressor in mice. The IKZF1 deletions were predominantly mono-allelic and were limited to the gene in 22 cases, identifying IKZF1 as the genetic target. Real-time quantitative PCR and FISH analysis confirmed SNP results and gene expression analysis showed that the genomic alteration in IKZF1 leads to a significant down-modulation of the transcript level. The deletions of IKZF1 in 12/25 patients were limited to an internal subset of exons (4-7), with a complete concordance between the extent of this deletion and the expression of the dominant-negative isoform Ik6 with cytoplasmatic localization and oncogenic activity. The deletions were restricted to highly localized sequences in introns 3 and 7. Heptamer recombination signal sequences recognized by the RAG enzymes during V(D)J recombination were located immediately internal to the deletion breakpoints, and a variable number of additional nucleotides (patient specific) were present between the consensus intron 3 and 7 sequences, suggestive of the action of terminal deoxynucleotidyl transferase (TdT) and aberrant RAG-mediated recombination. Deletion of IKZF1 was not identified in chronic phase CML (n=40) patients. In BCR-ABL1 ALL we also frequently observed a deletion of PAX5, CDKN2A, CDKN2B and BTG1. In conclusion, deletion of IKZF1 resulting in either haploinsuffciency and in the expression of the dominant negative isoform Ik6 is an important event in the development of BCR-ABL1 B-progenitor ALL. *European LeukemiaNet, AIL, AIRC, FIRB 2006, Fondazione del Monte di Bologna e Ravenna, Strategico di Ateneo.*

C043

PATTERN OF MENINGIOMA 1 GENE EXPRESSION IN DIFFERENT GENETIC SUBSETS OF ACUTE AND CHRONIC MYELOID LEUKAEMIAS AND ITS POTENTIAL USE AS A MARKER FOR MINIMAL RESIDUAL DISEASE DETECTION

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Meningioma 1 (MN1) gene overexpression has been reported in AML patients. The aim of the study was to characterize the patients presenting with MN1 overexpression and to establish if MN1 transcript could be a useful marker for MRD detection. 158 AML patients, 30 CML patients and 50 healthy donors were analyzed for MN1 expression by RQ-PCR. In 20 patients bearing a fusion gene transcript (FG), we analyzed MN1, WT1 and FG during follow-up and in 7 patients also NPM mutations has been quantified by RQ-PCR. The MN1 levels were extremely low in normal samples: median value of 136 MN1/104ABL copies in PB (range 9-300) and 254 in BM (range 80-500) and 12,9 (range 11-19) in CD34$^+$ cells. By contrast, about 50% of the AML samples with normal karyotype (NK) showed high MN1 expression with a median of 5136 copies/10^4 ABL copies (range 852-90230) and 6780, (range 1367-15900) in PB. All samples carrying the CBFbeta-MYH11 FG expressed a significantly higher amount of MN1 transcript as compared to controls (p<0,0001 in both BM and PB): median 46950, (range 2149-98000) in BM and 34500, (range 1400-67999) in PB. About 50% of the samples with AML1-ETO FG abnormally expressed MN1: median 16950, (range 3500-34000) in BM and 3475, (range 1260-56000) in PB. Finally, the APL samples expressed MN1 values comparable to those of healthy subjects in both BM and PB (p=0,05 and 0,08). No association between FLT3 mutations and MN1 was demonstrated. In contrast, MN1 is typically overexpressed in patients with NPM mutations. 36 out of 47 NPMc$^+$ patients were characterized by abnormal MN1expression. We were unable to confirm the correlation between EVI1 and MN1 expression (r=0,06) reported in literature. Finally, MN1 is not expressed in CP CML (10 cases) but is overexpressed during AP (n=10) and BC (n=10) (median 49100 and 62741 respectively). Finally MRD has been detected by measuring MN1, FG, WT1 and in 7 cases also NPM. MN1 expression always paralleled that of the FG. Furthermore, MN1 strictly paralleled WT1 and NPM in patients without any FGs. In all the cases MN1 rose at least two months before relapse. These data show that 47 % of patients with NK are characterized by abnormal MN1 expression. The overexpression is typical of AML with NPMc$^+$ and inv(16) and CML in AP and BC. MN1 could therefore represent a marker for MRD. Increased MN1 expression in the BM during follow up was always found to be

predictive of an impending hematological relapse.

C044

ALTERED MRNA EXPRESSION OF PAX5 ARE A COMMON EVENT IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. Pax-5 gene codifies for a transcription factor central to B-cell differentiation and function, expressed during the early stage of B-cell differentiation and alternatively spliced during B-cell development. In addition to the full-length isoform (Pax-5a), isoforms arising from the inclusion or exclusion of exons 2, 7, 8, and/or 9 have been described lacking the DNA-binding and the transactivating domains. These isoforms and their levels of expression increase during B-cell maturation. In particular, Pax-5b isoform (deleted of exon 2), resulting in protein with partial DNA-binding domain, is related with the differentiation stage. Recently, mutations of Pax-5 gene in pediatric B-ALL (38.9% in 192 B-ALL, Nature 2007) and in adult B-ALL (30% in 119 B-ALL, ASH 2007) have been described. We investigated Pax-5 gene isoforms in acute lymphoblastic leukemia. *Methods.* The expression pattern and the sequence of Pax-5 gene were investigated at diagnosis in leukemic cells from 37 adults and 2 children with B-cell precursor ALL. Pax-5a and Pax-5b expression levels were evaluated using quantitative PCR. To investigate expression pattern of Pax-5 in healthy donor (8 cases) we studied the Pax-5 mRNA in purified cell populations: purified B lymphocytes (6 samples of $CD19^+$ cells), hematopoietic progenitor cells (HPC) (6 samples of $CD34^+$ cells) and precursor-B-cells (2 samples of $CD34^+CD19^+$ cells). *Results.* Alternative splicing was found in 24/39 cases (61.5%). In 22 cases Pax-5b isoform was increased or exclusive; 16 had higher levels of isoforms with deleted exons 8 and 9, 4 had a novel isoform arising from the skipping of exon 5. Pax-5 point mutations were observed in 6 cases (15%): three mutations fall in exon 2 (53G>C, 197G>A, 76delG), one in the exon 6 (716G>A), one in exon 7 (836C>A), one in exon 10 (1133G>A). Control study showed only full-lenght isoform in HPC and progenitor-B-cells, while deleted isoforms were also present in mature B-lymphocytes. *Conclusions.* We provide the first evidence that Pax-5 gene is differentially regulated in the majority of cases of ALL. Absent or insufficient amount of full-length isoform, together with high levels of deleted-isoforms, are responsible for Pax-5 dysfunction in leukemic cells compared to their normal counterpart. We hypothesize that a premature event of alternative splicing in early stage of B-cell development may be cause of the block of differentiation which is part of neoplastic transformation. *Supported by a grant of Banco di Sicilia.*

C045

PAX5/TEL ACTS AS A TRANSCRIPTIONAL REPRESSOR ON B-CELL DEVELOPMENT GENES, AND ENHANCES MIGRATION TO CXCL12/SDF1 IN PRE-BI CELLS

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PAX5 is a transcription factor essential for B-cell development. Recently, it has been found as frequent target of abnormalities in childhood ALL (30% of B-cell precursor ALL cases), showing monoallelic loss, point mutations or chromosomal translocations. The role of these lesions is poorly understood. We previously cloned the PAX5/TEL fusion gene in a patient affected by B-cell precursor ALL with t(9;12) translocation. We investigated the functional roles of PAX5/TEL protein *in vitro*, in murine wild type pre-BI cells, derived from a wild type mouse and positive for B220, cKIT and CD19. We demonstrated that PAX5/TEL protein functions as a transcriptional repressor, down regulating PAX5- target genes encoding for proteins with a master role in B-cell development, such as CD19, B220, BLNK/SLP65, MB-1/CD79a, immunoglobulin mu-chain, but also CD44 and CD11a. In addition, PAX5/TEL down regulates FLT3 expression, and it does not activate MCSFR gene. Moreover, the fusion protein represses TEL-target genes, such as KLF4, PHLDA1 and SPHK1. In $PAX5^{-/-}$ pre-BI cells, PAX5/TEL did not restore CD19 expression. Comprehensively, these findings suggest that the fusion protein functions as a dominant repressor of transcription on PAX5- and TEL- target genes. In several hematopoietic malignancies, the expression of CXCR4 and the specific migration to CXCL12/SDF1 have an important role in tumour proliferation, and we demonstrated that PAX5/TEL enhances cell migration towards this chemokine, with over-expression of its receptor. Together with previous evidences on the PAX5/TEL capacity to overcome IL-7 withdrawal and to interfere with TGFbeta1 pathway, we conclude that PAX5/TEL induces resistance to apoptosis and interferes with the processes of B-cell differentiation and migration. Taken together, these phenomena likely represent key events in the process of B-cell transformation.

C046

ACTIVATION-INDUCED CYTIDINE DEAMINASE IS ABERRANTLY OVEREXPRESSED IN BCR-ABL1 POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA CELLS AND PROMOTES DNA-SINGLE STRAND BREAKS

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The Philadelphia chromosome (Ph) encodes the oncogenic BCR-ABL1 kinase and defines a subgroup of ALL with a particularly unfavorable prognosis. ALL cells derived from B cell precursors typically carry rearranged immunoglobulin heavy chain (IGH) variable (V) region genes devoid of somatic mutations. Somatic hypermutation is restricted to mature germinal center B cells and depends on expression of activation-induced cytidine deaminase (AID), which introduces single-strand breaks into target DNA. Since, at much lower frequency AID can also target non-Ig genes and may even act as a genome-wide mutator, we investigated whether AID was expressed in Ph^+ ALL and blast crisis chronic myeloid leukemia (CML) patients. On 35 adult Ph^+ ALL patients, we detected AID mRNA and protein in 16 (46%), moreover AID expression was also found in blast crisis B-lymphoid lineage CML patients (n=2) but not in myeloid lineage (n=5) or in chronic phase CML (n=30). AID expression correlated with the BCR-ABL1 transcript levels and with the frequency of mutations within the BCR-ABL1 kinase domain that confer resistance to the BCR-ABL1 kinase inhibitors and disappeared after treatment with imatinib at the time of remission. To investigate whether AID introduces DNA-SSB in Ph^+ ALL, we performed a genome wide analysis by 250K NspI single nucleotide polymorphism (SNP) array (Affymetrix Inc., USA) which is able to detect at high resolution and throughout the genome copy number abnormalities. We identified region of high level amplification and homozygous deletion in all patients. Overall, deletions outnumbered amplifications 3:1 and on average, we found 10 lesions per case, with a median of 6 losses and 2 gains. Patients who expressed AID had a higher number of alterations respect to patients who were AID negative (median copy number alteration was 8.5, range 4-22, versus 4, range 1-10, respectively, $p<0.004$). Recurring copy number abnormalities were identified in genes with an established role in leukemogenesis such as CDKN2A, CDKN2B, GADD45, PAX5, ETV1, BTG1 and MDS1. In particular, we found that the pattern of AID expression correlated with a high frequency of DNA single-strand breaks within the tumor suppressor genes CDKN2A and CDKN2B, which were found in B cell lineage but not myeloid lineage subclones of blast crisis CML. These findings show that AID can act as a mutator outside the Ig gene loci in Ph^+ ALL cells prommoting genetic instability in leukemia cells. *Supported by European LeukemiaNet, AIL, AIRC, FIRB 2006, Fondazione del Monte di Bologna e Ravenna, Strategico di Ateneo.*

C047

MUTATIONAL ANALYSIS OF NOTCH1 AND FBW7 IN T-ALL. A GIMEMA STUDY.

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Background. NOTCH1 activating mutations concur in the development of the majority of T cell acute lymphoblastic leukaemia (T-ALL) and indeed, therapy based on gamma-secretase inhibitors targets the NOTCH1 pathway. Most mutations truncate the NOTCH1 C-terminal (PEST domain) where the FBW7 gene, ubiquitin ligase, targets NOTCH1 for ubiquitination and degradation. Aims and methods. To analyze NOTCH1 and FBW7 mutational status in 27 adult patients with T-ALL we performed mutational analysis with PCR-based Denaturing High-Performance Liquid Chromatography (DHPLC) using a WAVE-MDTM System (Transgenomic, Omaha, NE). For NOTCH1 we analyzed Exons 26 (HD-N domain), 27 (HD-C domain), and 34 (PEST domain), and for FBW7 we analyzed Exons 8-9 (WD40 repeats III and IV), which are a NOTCH1 interaction site. *Results.* In 24/27 (88.8%) patients the NOTCH1 gene profile was altered on DHPLC. In these 24 cases, direct sequencing indicated 9 (37.5%) had known polymorphisms and 15 (62.5%) were mutated, for a total of 19 mutations (8 new). Mutations were detected in the HD domain in 6/15 (40%), in the PEST domain in 7 (46.6%) and in both domains in 2 (13.3%). In 5/27 cases (18.5%) the FBW7 gene profile was altered on DHPLC. Direct sequencing indicated mutations were in WD40 repeats III in 2 cases, mutations in the WD40 repeats IV in 2 and in both domains in 1. None of the T-ALL samples bearing FBW7 mutations had mutations in the PEST domain of NOTCH1 but one case had a mutation in the HD domain of NOTCH1. Table 1 shows details of all mutations in the 27 patients. *Conclusions.* NOTCH1 protein stability appears to be a major regulator of intracellular signalling thresholds. Abrogation of the NOTCH1 degradation machinery predisposes cells to transformation. This study confirms NOTCH1 mutations were present in more than 50% of adult T-ALL. FBW7 gene mutations appeared to occur as an independent event but were found concomitantly with NOTCH1 mutations in one patient. Previous studies on T-ALL cell lines harbouring NOTCH1 and FBW7 mutations showed resistance to gamma-secretase inhibitors. Our patient with both mutations, relapsed 9 months after an initial complete remission obtained with the GIMEMA protocol ALL 0904. The incidence of concurrent NOTCH1 and FBW7 mutations needs to be assessed in a much larger series of patients in order to determine the best therapeutic strategy for them.

Table 1. NOTCH1 and FBW7 mutations in T-ALL patients.

Case no.	NOTCH1 mutations (domain/class/type)	Amino acids	FBW7 mutations (domain/class/type)	Amino acid
1	HD / PM / c.4802T>C	p.L1601P		
2				
3			repeats III / PM / c.1543G>A	p.R465H
			repeats IV / PM / c.1578G>A	
4	HD / DEL-INS / c.4825_4843del18ins12	p.R1609_Q1614>PPKA		
5				
6				
7	HD / PM / c.5036T>C	p.L1679P	repeats III / PM / c.1542C>T	p.R465C
8				
9	PEST / INS / c.7329ins(CACCC)	p.V2444>HfsX35		
10	HD / DEL / c.4735_4737delGTG	p.V1576del		
	PEST / DEL-INS / c.7330_7330G>(CCA)	p.V2444>PfsX35		
11			repeatsIV / PM / c.1585G>A	p.R479Q
12				
13			repeats III / PM / c.1542C>T	p.R465C
14	PEST / DEL-INS / c.7570_7570(CG)>(TTC)	p.S2524>F		
15	PEST / DEL / c.7389delC	p.P2463fsX15		
16				
17	HD / PM / c.5029G>T	p.V1677>F		
	PEST / INS / c.7330_7331ins11	p.V2444>Pfsx36		
18	PEST / INS / c.7330_7331ins(TAG)	p.dupV2444		
19				
20	HD-N / PM / c.4864G>C	p.G1622>R		
	HD-C / PM / c.5036T>C	p.L1679P		
21				
22	PEST / DEL / c.7544_7545delCT	p.P2515SfsX4		
23	PEST / INS / c.7329-7330ins16	p.D2443Lfsx68		
24	PEST / INS / c.7360-7361ins(CG)	p.R2474>VfsX5		
25	HD / PM / c.5101G>C	p.A1701>P		
26	HD / PM / c.5164 G>A	p.V1722M		
	HD / PM / c.7473 C>A	p.Y2491*Fs		
27			repeatsIV / PM / c.1663 G>T	p.R505L

HD, heterodimerization domain, HD-N, heterodimerization domain N terminal, HD-C, heterodimerization domain C terminal, PEST, polypeptide enriched in proline, glutamate , serine and threonine, PM, point mutation; DEL, deletion; INS, insertion

C048

THE GENERATION OF SENSITIVE, PATIENT SPECIFIC MOLECULAR PROBES IS FEASIBLE IN MOST ADULT ALL PATIENTS AND ALLOWS THE DEFINITION OF A RISK ADAPTED POSTCONSOLIDATION TREATMENT STRATEGY

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Background. Several studies have shown the importance of a molecular evaluation of minimal residual disease (MRD) to predict outcome in childhood acute lymphoblastic leukemia (ALL) but in the setting of adult ALL, only a few studies define a risk adapted treatment strategy on the basis of MRD results. *Aims.* To generate at least 1 sensitive allele specific oligonucleotide (ASO) probe derived from TCR and IG gene rearrangements for RQ-PCR evaluation of MRD in adult ALL patients lacking a translocation derived chimeric transcript. The availability of sensitive molecular probes were instrumental for the definition of risk adapted post consolidation therapy. *Methods*. A total of 310 patients (180 B and 107 T-lineage ALL, 5 B and 18 T-Lymphoblastic Lymphomas) enrolled in a Phase II clinical study (ALL 09/2000) of the Northern Italy Leukemia Group (NILG) were evaluated following the ESG-MRD-ALL (European Study Group on MRD detection in ALL) methodology. Clonal TCR and IG gene rearrangements were identified by PCR, heteroduplex analysis and sequencing. Two or more ASO primers were designed on each specific junctional region and tested at different temperatures to reach a sensitivity suitable for MRD evaluation on serial dilution of diagnostic material by RQ-PCR. *Results.* TCR gamma (TCRG) rearrangements were detected by PCR in 49% (n=88) of B-lineage ALL and in 73% (n=77) of T-lineage ALL, while TCRD rearrangements in 40% (n=72) and in 47% (n=50) of B- and T-ALL respectively. IgH rearrangements were detected in 59% (n=106) of B-lineage and in 4% (n=4) of T-lineage ALL. IGK-Kde rearrangements were investigated only in B-lineage ALL and the positivity for clonal bands was 32% (n=57). TCR beta clonality was studied only in T-ALL and it was found in 65% (n= 64) of cases. The distribution of the clonal rearrangements were similar in the lymphoblastic lymphomas. On a total of 484 tested ASO primers we obtained a sensitivity of 10-5 in 48% (n=232), 10-4 in the 35%(n= 167) and 10-3 in 16% (n=79). Overall, we were able to monitor MRD in 81% of the studied cases, with 2 or 1 ASO probes in 49% (n=135) and 32% (n=90) of cases, respectively. *Conclusions.* In combination with probes generated on translocation derived chimeric genes, more than 90% of adult ALL enrolled into the NILG ALL 09/2000 protocol were suitable for a molecular evaluation of MRD. Therefore, our data show the feasibility of a MRD based definition of risk adapted post consolidation treatment strategy in adult ALL.

Chronic Myeloid Leukemia and Myeloproliferative Disorders (II)

C049

EFFICACY AND SAFETY OF DASATINIB IN CML/ALL PH1+ IMATINIB RESISTANT PATIENTS IN A COMPASSIONATE USE PROGRAM

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Dasatinib demonstrated the greatest potency *in vitro* against ABL kinase activity of all the second-generation ABL kinase inhibitors (TKi). Its activity against nearly all imatinib-resistant BCR-ABL mutations has been confirmed clinically. From May 2006 to June 2007 93 pts with CML imatinib resistant or intolerant and 33 ALL Ph1+ with same characteristic have been treated with dasatinib in a compassionate use. Forty-five patients were affected from CML-Chronic phase (CP), 19 accelerate-phase, 29 blast crisis and 33 were ALL Ph1+. Dasatinib was administered 70 mg BID, and dose escalation to 100 mg BID and reduction to 50 mg or 40 mg BID were allowed for inadequate response or adverse events (AEs), respectively. Imatinib resistance was primary (39 pts) and acquired (68 pts), median age was 55 years (range 17-76). The median time from CML/ALL Ph+ diagnosis to enrolment in the compassionate use was 48 months. All pts received prior imatinib at median dose 439mg/d for CML-CP, 449 mg for CML-advanced phases (AdP) and 600 mg for ALL Ph+, for a median time of 1191 days for CP, 1020 for AdP and 277 for ALL Ph+. The best previous response to imatinib was CCyR in 27% of CML-CP, 42% in ALL Ph1+, and 31% in CML-AdP. Prior treatment for CML included IFN-α in 2 ALL Ph1+ pts (1,5%) and in 46 CML pts (36%), chemotherapy in 44 ALL Ph1+ pts (35%) and stem cell transplantation in 7 ALL Ph1+ pts (5,5%) and in 9 CML pts (7%). Twenty-seven patients (21,4%) received other TKi before dasatinib administration. With a median follow-up of 12 months, the CHR rate was 82% for CML-CP, 75% for CML-AP, 58% for ALLPh1+, respectively. The CCyR rate was 36% in CML-CP, 17% in AdP and 18% in ALLPh1+. The median dose of dasatinib has been 111 mg for CML-CP, 108 mg for CML-AP and 140 mg for ALLPh1+. Thirty-five pts (27,7%) required a dose interruption; 4,7% were due to hematologic toxicity and 11% due to non-hematologic toxicity. Fifty pts remain on treatment with a median of 464 days; 41 had progression of disease. AEs (all grades) considered drug related included headache 4,2%, diarrhea 10,4%, nausea 16%, fatigue 10,4%, rash 8,3%, edema 2,1%, dyspnea 6,3%, pleural effusion 9%, myalgia 7%, arthralgia 4,2%, pneumonia 6,3%, and gastrointestinal hemorrhage 1%. The PFS at 12 mo was 86% in CML-CP, 51,4% in CML-AP and 24% in ALLPh1+. OS at 12 mo was 92% for CML-CP, 63,4% for CML-AP and 31% for ALLPh1+. An update with molecular response data and a durable stable disease condition will be considered.

C050

IDENTIFICATION OF CANDIDATE GENES SUSTAINING BCR-ABL ONCOGENIC SIGNALLING AND CML PROGRESSION THROUGH A GENETIC TOOL BASED ON HUMAN BCR-ABL TRANSGENIC DROSOPHILA MELANOGASTER

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Although the role of Bcr-Abl in the pathogenesis of Chronic Myeloid Leukaemia (CML) is well established, the mechanisms responsible for CML progression are largely unknown. The aim of the study was to perform a genetic screening to identify new pathways leading to CML resistance and progression and to provide a powerful tool allowing a wide screen of drug libraries. We developed a genetic model based on transgenic human p210 Bcr-Abl Drosophila melanogaster (Dm) generating two different fly lines expressing either h-p210 wt or carrying the T315I mutation, in a tissue specific manner such as fly eyes or lymph gland, which represents the Dm hematopoietic system. This approach allows to identify genetic pathways leading to disease without requiring a priori knowledge of the gene function. A wide modifier screen was performed using P-elements which randomly insert into the genome, altering the gene function. The heterogeneous mutagenized fly population was first screened using eye phenotype as read-out system and candidate genes were then identified by inverted PCR. In addition, a drug screening was performed by feeding flies with drugs. Bcr-Abl expression results into a glazed phenotype correlated with the amount of p210 protein. The P-element insertions generated a heterogeneous progeny whose phenotype is influenced by the affected genes: a first group of flies displayed a more aggressive phenotype since they harbour mutated genes encoding for Bcr-Abl negative regulator while a second group displayed a mild phenotype most likely due to a mutation in a gene encoding for a downstream effector of the oncogenic signalling. Among the genes identified, PI3K loss of function results into a phenotype improvement thus supporting the tool effectiveness. In addition, genes known to modify the phenotype of dAbl mutant flies were also able to modulate Bcr-Abl phenotype. The genes identified by wide-eye screening as Bcr-Abl modifier, were also able to induce alterations in the lymph gland. Data obtained with Dm were confirmed in CML cells by overexpressing or silencing genes previously identified. Finally we have set up a rapid method for drug testing based on wt and T315I/Bcr-Abl phenotypes rescue induced by several TKs inhibitors. Using Dm we identified a number of genes probably involved in CML progression and IM resistance and set up an easy and rapid tool for drug libraries screening which will allow to identify molecules able to silence T315I/Bcr-Abl TK activity.

C051

NOT REGULATORY CD4+/CD25HIGH+/FOXP3+ AND CD4+/PERFORIN+ PEPTIDE-SPECIFIC T CELLS ARE INDUCED BY P210-DERIVED PEPTIDE VACCINATIONS IN CHRONIC MYELOGENOUS LEUKEMIA PATIENTS

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Previously, vaccinations of chronic CML patients with CMLVAX100 (5 p210-b3a2 breakpoint derived peptides) plus GM-CSF were shown to induce an evident, durable peptide-specific CD4+ T cell proliferative response in most of patients. The response is mainly due to a 25 mer long breakpoint peptide (b3a2-25) included in the vaccine. About 60% of 32 CML patients vaccinated while on imatinib, showed a reduction of their minimal residual disease after immunization and about 25% of them achieved even a complete molecular response. To investigate the contribute of peptide-specific CD4+ T cells in antitumor activity, in 10 vaccinated patients we further characterized proliferating CD4+ cells for the co-expression of CD25, perforin and FOXP3 molecules by flow-cytometry. After additional *in vitro* stimulation with b3a2-25 peptide, we observed the increase of two main CD4+ T cells subsets: 1) a small-size CD4+/perforin+ T cell population (raising from a median of 1% to 2.3%) with potential cytotoxic activity 2) a large-size CD4+/CD25^{+high}/ FOXP3+ T cell population (raising from 0.29% to 5.65%) with potential regula-

tory function even though the large size and the high proliferative activity would argue against it. Thus, in 3 selected vaccinated patients we expanded the $CD4^+/CD25^{+high}/FOXP3^+$ population with further *in vitro* b3a2-25 peptide stimulations and we performed a functional test to measure its potential regulatory activity. Hence, we evaluated the ability of expanded b3a2-25 peptide specific $CD4^+/CD25^{+high}/FOXP3^+$ T cells to inhibit the growth of CFSE labelled normal subjects naïve $CD4^+$ T cells stimulated with autologous CD3-depleted APCs and antiCD3 plus antiCD28 MoAbs. In our experimental conditions, naïve CFSE $CD4^+$ T cells equally proliferated in the presence of b3a2-25 specific $CD4^+/CD25^{+high}/FOXP3^+$ T cells, no peptide $CD4^+$ cells or a control peptide stimulated $CD4^+$ cells and the rate of proliferation was similar to the one observed in co-culture experiments with allogeneic normal $CD4^+$ cells. Our data showed that vaccination of CML patients with CML-VAX100 are followed by the proliferation of b3a2-25 peptide-specific $CD4^+$/perforin$^+$, but mainly of b3a2-25 specific $CD4^+/CD25^{+high}/FOXP3^+$ T cells. The latter display no regulatory activity despite their phenotype. We are now planning to evaluate whether and how these peptide-specific $CD4^+$ T cells exert a direct antitumor activity against CML cells.

C052

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

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Interferon Regulatory Factor 5, is a member of the Interferon Regulatory Factors (IRFs) family. This family comprise helix-turn-helix transcription factors which are pivotal regulators of the immune response against viral infections and cancer. Initially studied as inducers or suppressors of type I IFN production, IRFs are now known to bind an extensive number of gene promoters, functioning as transcriptional activators and/or transcriptional repressors. Recent studies have demonstrated that they play important roles in the regulation of cell growth, differentiation, oncogenesis and apoptosis. In fact, IRF-5 has tumor suppressor properties, it is a direct target of p53 and its expression is silenced in different hematologic malignancies. In addition, IRF-5 induces G2-M cell cycle arrest and regulates the expression of bak, caspase-8, Bax and p21 genes. Aim of this research was to study the functional relationship between IRF-5 and BCR-ABL in Chronic Myeloid Leukemia (CML). We utilized three BCR-ABL-positive cell lines: K562, KCL22 and KYO-1 and BCR-ABL-negative HL-60 cells as a control. We found that IRF-5 is expressed in primary BCR-ABL-positive cells as well as in immortalized CML cell lines. In addition, co-immunoprecipitation experiments showed that IRF-5 and BCR-ABL displays a direct interaction which is dependent on BCR-ABL tyrosine kinase activity. Interestingly, we have detected high levels of IRF-5 tyrosine phosphorylation in CML cells but not in the HL60 control and such a phosphorylation decreases after treatment with Imatynib Mesylate (IM), a semi-specific inhibitor of BCR-ABL tyrosine kinase activity. Finally, cell fractionation experiments showed that, in CML cell lines, IRF-5 localization is radically modified by IM treatment, suggesting that BCR-ABL might alter the regulation of IRF-5 nuclear-cytoplasmic shuttling. In summary our findings show that the functional interaction between BCR-ABL and IRF-5, which depends on BCR-ABL tyrosine kinase activity, prevents IRF-5 to translocate in the nucleus and consequently to exert its function.

C053

GDF15 SERUM EVALUATION IN MYELODYSPLASTIC AND MYELOFIBROTIC PATIENTS AS A MARKER OF INEFFECTIVE ERYTHROPOIESIS

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Background. The small liver peptide hepcidin is the principal regulator of iron homeostasis in humans through the binding and the subsequent degradation of ferroportin both in enterocytes and macrophages. Growth differentiation factor 15 (GDF15) is a member of the transforming growth factor-beta family released by erythroblast progenitors during differentiation. Recently it has been described as a potential hepcidin regulator in thalassemia patients who show a significant increase of serum GDF15 concentration. Little is known about iron metabolism in myelodysplastic syndromes (MDS) and primary myelofibrosis (PM) which are characterized by a high degree of ineffective erythropoiesis. *Aims.* to evaluate serum GDF15 concentrations in a population of MDS and PM patients at diagnosis or sequentially before and during iron chelation treatment (ICT). *Methods.* 30 peripheral blood samples were collected from patients affected by MDS (12 RA, 10 RAEB, 4 unclassified) and primary myelofibrosis (4). Median serum ferritin level was 500 ng/mL, median haemoglobin value was 8,5 g/dL. 18 out of 30 patients were untransfused, 5 subjects were analyzed both before and during ICT. Serum GFD15 evaluation was performed by ELISA. Serum hepcidin evaluation was performed by SELDI-TOF-MS. The results were compared to 10 healthy controls and 30 thalassemic patients. *Results.* Serum GDF15 values in MDS patients with erythroid dysplasia were significantly increased compared to healthy controls (median value 2537 versus 206, $p<0,001$ by Mann-Whitney test) and significantly different compared to thalassemic patients (median value 6769, $p=0,04$). By contrast, no difference was found between values detected in MDS patients without erythroid dysplasia and healthy subjects. Serum hepcidin levels in MDS untransfused patients were decreased compared to healthy controls. Interestingly, regression analysis shows no correlation between GDF15 levels and, respectively, hepcidin, ferritin and haemoglobin values. *Discussion.* Serum GDF15 is markedly increased in a cohort of MDS and PM patients with erythroid dysplasia but not in cases with only megakaryocytic or myeloid dysplasia compatible with a marker of ineffective erythropoiesis. We failed to observe any correlations between GDF15 and hepcidin levels in untrasfused MDS patients but our data need to be confirmed on a larger cohort of patients.

C054

CCL6 AND CCL9 CHEMOKINES AND INTERFERON-MEDIATED IMMUNE PROTECTION IN PHILADELPHIA POSITIVE CHRONIC MYELOID LEUKEMIA

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Despite the indisputable success of TKIs in prolonging the survival of CML patients, these agents are rarely curative and treated patients can relapse with drug-resistant disease. There is a recognized need for additional approaches in the CML treatment. Biological therapies are one alternative strategy with proven efficacy. Interferon alpha (IFN alpha) induces durable complete cytogenetic responses, even off therapy, in a subset of patients. The downstream molecules through which IFN alpha induces its effects and how these connect to pathways deregulated in CML remains poorly understood, but evidence supports an immune mechanism. The Type I Interferons regulate the expression of the tumor suppressor Interferon Consensus Sequence Binding Protein (ICSBP) in bcr-abl transformed cells and, as shown previously for ICSBP, they induce a vaccine-like immunoprotective effect in a murine model of bcr-abl induced leukemia. However, how ICSBP is regulated in CML cells

and the downstream pathways through which it induces an anti-leukemic response remains unclear. We show that IFN alpha upregulates ICSBP expression in bcr-abl transformed cells and can substitute for ICSBP overexpression in protecting mice from leukemia. The protection conferred by the IFNs requires intact immunity. Type I IFNs rescue mice from leukemias caused by imatinib-resistant BCR-ABL mutants commonly found in CML patients, including the T315I. Using transcriptional profiling and semi-quantitative PCR, we identify the chemokines CCL6 and CCL9 as genes prominently induced by the Type I Interferons and ICSBP, and demonstrate that these immunomodulators are required for the immunoprotective effect of ICSBP expression. Both CCL6 and CCL9 chemokines are necessary, although not sufficient, for ICSBP to confer immune protection against a BCR-ABL induced leukemia in mice. Human IFN alpha induces the expression of CCL15 and CCL23, the human orthologues of CCL6 and CCL9, respectively. We used qRT-PCR to determine the expression level of CCL23 in PBMCs isolated from CML patients treated with IFN alpha therapy and we found that induction of CCL23 stratifies patients with a favorable clinical response. Insights into the role of these chemokines in the anti-leukemic response induced by interferons suggest new strategies for immunotherapy: co-administration of CCL15 and CCL23 might be a valuable adjunctive therapy to the peptide vaccines that are currently in clinical development as CML therapies.

C055

NILOTINIB 400 MG BID AS FIRST LINE TREATMENT OF CHRONIC MYELOID LEUKEMIA IN EARLY CHRONIC PHASE: RESULTS OF A PHASE 2 TRIAL OF THE GIMEMA CML WORKING PARTY

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Imatinib (IM) 400 mg daily is the standard treatment for chronic myeloid leukemia (CML) in early chronic phase (ECP): the results of the IRIS trial have shown a 72 months estimated overall survival of 95%; the complete cytogenetic response (CCgR) rates were 25%, 51%, 69% and 87% at 3, 6, 12 and 60 months, respectively. Nilotinib (NL), a second generation TKI, has a higher binding affinity and selectivity for Abl with respect to IM and is highly effective in IM resistant patients, across every disease phase. Among 320 patients in late chronic phase, resistant or intolerant to IM, who received NL 400 mg BID for at least 6 months, the rates of major and complete cytogenetic response were 56% and 40%, respectively, with very few progressions. Based on these data, NL is currently registered for the treatment of CML patients intolerant or resistant to IM, but it may compete with IM for the frontline treatments of ECP patients. To investigate the therapeutic efficacy and the safety of NL 400 mg BID in ECP Ph-positive CML patients the GIMEMA CML Working Party planned an open-label, single stage, multicentric, phase II study, open to accrual in June 2007. The primary endpoint is the CCgR rate at 1 year; the kinetic of MR is studied by Q-PCR, baseline and after 1, 2, 3, 6, 9 and 12 months form starting the treatment. 75 patients have been enrolled from 18 Centres between June, 2007 and February, 2008. The median age was 51 years (range 18-83); 55% low, 34% intermediate and 11% high Sokal risk. Median follow-up is currently 6 months (range 1-11) and thirty-six patients are fully evaluable for response at 3 months: 35/36 achieved a CHR; 31/36 reached a MCgR (86%) with 29/36 in CCgR (81%). The MMR rate (BCR-ABL:ABL <0.1% according to the International Scale) among the CCgR patients only was 38% (12/29 CCgR patients). Adverse events grade III/IV: hematologic toxicity was recorded in 2 pts (6% - only 1 epysode of grade IV neutropenia); non-hematologic AEs in 4 pts (11% - no grade IV) and biochemical abnormalities in 11 pts (31% - mainly transient increased bilirubin or transaminases; no grade IV). After 3 months, 70% of patients are still on treatment with NL 400 mg BID, while 30% is assuming 400 mg OAD. The results that have been achieved so far strongly support the hypothesis that in ECP Ph-positive CML patients the response to NL may be faster than the response to IM. *Acknowledgements: European LeukemiaNet, COFIN, University of Bologna and BolognAIL.*

C056

GATA1 IS UP-REGULATED IN PATIENT AFFECTED BY ESSENTIAL THROMBOCYTHEMIA AND POLYCYTHEMIA VERA BUT NOT IN PRIMARY MYELOFIBROSIS OR CHRONIC MYELOGENOUS LEUKEMIA

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In vitro studies, murine models, and sporadic reports on human samples suggest that a connection exists between the transcription factor GATA1 and myeloproliferative disorders (MPDs). We measured the GATA1 expression to determine the differences among the chronic myeloproliferative disorders in order to prove a role of GATA1 in the pathogenesis of the diseases and propose it as a new molecular marker useful in diagnosis and molecular follow-up. We collected 92 bone marrow aspirates from newly diagnosed patients affected by myeloproliferative disorders (MPDs) or acute myeloid leukaemia (AML) according to the WHO criteria: 46 essential thrombocitemia (ET) patients, 14 polycythemia vera (PV), 12 chronic phase of chronic myeloid leukaemia (CML), 10 primary myelofibrosis (PMF) and 10 AML. Six aspirates from healthy donors, and 7 from patients with reactive myeloproliferation (4 idiopathic thrombocytopenic purpura and 3 secondary erythrocytosis) were used as controls. We performed Syber-Green Real Time PCR for GATA1 detection. The relative GATA1 quantification was calculated according to the DCt method with GAPDH as internal control. We found GATA1 overexpressed in ET (median 89.48; range 7.03-282,636.84) and PV patients (median 167.73; range 68.12-270.6), but not in IMF (median 3.8; range 0.13/12.12), or CML (median 2.1; range 0.39/6.82), comparing with 6 aspirates from healthy donors (median 1.109; range 0.06-20.73), (p<0,003) and with reactive thrombocytosis (median 0.8; range 0.53-0.93) or erythrocytosis (median 2.42; range 0.8-2.7) (p<0,001). In the ET group, no significant differences were found in GATA1 expression in patients harbouring a JAK V617F mutation (median 77.64; range 1.46/282636) and in those with wild type JAK2 alleles (median 213; range 9.69/71047) (p>0.3). As previously described, the GATA1 expression in patients affected by AML may be variable. In our AML patients the GATA1 level was significantly lower then normal controls (p<0.005) and absolutely reduced comparing to ET and PV patients (p<0.0001). GATA1 expression could be identified as a new approach for better understanding the molecular mechanisms of MPDs. It might become an additional marker of the diseases, useful to detect the residual clone after and during treatment of ET and PV, especially when there are no other molecular markers for following the minimal residual disease, such as in ET patients without the JAK2 mutation. Finally, we could imagine GATA1 as a new possible target for specific treatment.

Transplantation, Cell Therapy and Microenvironment

C057

EFFICIENT DIFFERENTIATION OF PRIMARY AND SECONDARY ACUTE MYELOID LEUKEMIAS INTO FUNCTIONALLY COMPETENT LEUKEMIC DENDRITIC CELLS TO IMPROVE THE GRAFT VERSUS LEUKEMIA EFFECT

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In recent years, the intensification of chemotherapy protocols has led to an improvement of survival only in selected sub-populations of good-risk patients affected by acute myeloid leukemia (AML). The outcome of patients affected by high-risk de-novo and secondary AML has unfortunately unchanged across the last decade. Although sensitivity of leukemia to immune control is clearly shown by results of allogeneic hematopoietic stem cell transplantation (allo-HSCT) and donor lymphocyte infusions, the low rate of persistent remissions emphasize the need of more specific and effective immunotherapeutic approaches. Myeloid blasts are poor antigen presenting cells (APC), but have the unique ability to differentiate into dendritic cells (DCs), offering a model in which the most potent APC and the tumor cell coincides. Theoretically, such leukemic dendritic cells (LDC) have the potential to boost the graft-versus-leukemia (GvL) effect of allo-HSCT. Unfortunately,current protocols for LDC differentiation, based on blasts exposure to cytokine cocktails, are limited by a low (>30%) efficiency. The aim of this study was to evaluate the ability of the calcium ionophore A23187 in combination with IL-4, to overcome cytokine resistance of primary and secondary AML, thus generating functionally competent LDC, able to boost GvL. In a cohort of 14 AML patients, we observed that a short (48 hrs) exposure to A23187+IL4 induce LDC differentiation of a large proportion (12/14) of de novo and secondary AML blasts. Compared to original blasts, LDC showed a significant up-regulation of several molecules involved in the immunological synapse, such as CD86, CD80, HLA-DR, CD54 and CD58, and proved negative for the expression of the tolerogenic marker PDL1. This favourable phenotypic profile correlated with a high T cells stimulatory capacity, that proved similar to that of healthy mature DCs. Furthermore, LDCs differentiated upon calcium ionophore exposure, maintained the expression of several disease markers (ie: CD34 and CD117) and of the leukemic antigen WT1. Most importantly, stimulation of HLA-identical and haploidentical allogeneic T lymphocytes with LDC allowed a rapid isolation and expansion of leukemia-reactive effectors ($gIFN^+$) and central memory cells. The immunostimulatory capacity of LDC clearly correlated with the level of LDC maturation. Interestingly, with this protocol we directly induced a mature $CCR7^+$ $CD83^+$ phenotype in 44% of LDC, with the highest frequency observed among AML blasts secondary to myelodisplastic syndromes, suggesting the existence of molecular factors committing blasts from secondary leukemias to DC lineage. The efficacy of A23187+IL4 in differentiating both primary and secondary leukemic blasts into LDC provides the rational for novel adoptive immunotherapeutic approaches to treat high risk AML.

C058

MOLECULAR REMISSION AFTER REDUCED INTENSITY ALLOGENEIC TRANSPLANTATION IN CHRONIC LYMPHOCYTIC LEUKEMIA: A SURROGATE MARKER OF THE GVL EFFECT THAT PREDICTS SURVIVAL AND OVERCOMES POOR PROGNOSTIC FACTORS

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Reduced intensity allogeneic stem cell transplantation (RIC alloSCT) can lead to 50% of progression-free survivors in poor risk relapsed chronic lymphocytic leukemia (CLL). The aims of this study were to assess the rate of molecular remission (MR) and the correlation with relapse risk, and clinical or biological factors. Twenty-nine patients in complete remission (CR) and with an available immunoglobulin heavy chain gene rearrangement (IgH) were monitored for minimal residual disease (MRD) by PCR. Seventy-five percent had an unmutated IgH; 6 patients had a 17p deletion. Median age at transplant was 60 years (range, 44-69). All patients had a relapsed disease after a median number of previous chemotherapy of 3, 29% of patients failed an autologous transplant and 38% were chemorefractory. Donors were HLA identical (n=21) or haploidentical (n=2), or matched unrelated (n=6). Molecular monitoring was performed by nested-PCR on bone marrow using patient-specific primers. For real-time PCR relative quantification was estimated by using a FR3-derived probe. Nine patients (31%) were PCR-negative at the median follow-up of 20 months (range, 6-71) and none of them relapsed. Seven patients (24%) showed a mixed pattern of PCR: 5 of them are in CR at a median follow-up of 30 months (range, 7-61). Thirteen patients (45%) were always PCR-positive: 7 of them relapsed after a median time of 9 months, 4 patients are alive and in CR after a median follow-up of 17 months (range, 3-24). The cumulative incidence of relapse based on the MRD status within the first 6 months after transplant was significantly different between PCR-negative/mixed and PCR-positive patients (p= 0.03). Two-year DFS was 93% and 25% for PCR-negative/mixed and PCR-positive patients respectively (p=<0.01). In 3 PCR-positive patients that did not relapse a decreasing tumor load was detected by real-time PCR; in 2 PCR-positive patients the tumor load increased and both patients subsequently relapsed. Disease status before transplant, the number of previous lines of therapy, donor type and the occurrence of GVHD did not correlate with the achievement of MR. 50% of 17p deletion patients achieved a mixed/negative PCR status. The telomere length (p=0.52) and the IgH-derived epitope binding affinity on patient HLA class I and II (p=0.29) did not predict the molecular outcome. In summary, the achievement of MR predicts a better survival in poor prognosis and relapsed patients.

C059

MACROPHAGE INFLAMMATORY PROTEIN-3ALFA/CCL20 AND ITS RECEPTOR CCR6 ARE OVEREXPRESSED IN THE BONE MICROENVIRONMENT AND INVOLVED IN OSTEOCLAST FORMATION IN MULTIPLE MYELOMA PATIENTS

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Osteoclast (OC) activation in multiple myeloma (MM) is primarily due to the imbalance of the critical osteoclastogenic system RANKL/OPG in the bone microenvironment. Recent evidences indicate that chemokines, small chemoattractant proteins involved in cancer cell homing, may contribute to osteoclast formation and activation as shown for CCL3/MIP-1alfa in MM. The expression of the chemokine CCL20/MIP-3alfa and its receptor CCR6 by multiple myeloma (MM) and bone microenvironment cells and their potential relationship with osteoclast (OC) formation and the development of osteolytic bone lesions in MM patients has been investigated in this study. First we found that MM cells rarely produce CCL20/MIP-3alfa but up-regulate its production by bone marrow (BM) osteoprogenitor cells and osteoblasts in co-culture with the involvement of soluble factors as IL-1beta, TNFalfa and IL-6 that had a synergistic effect on CCL20/MIP-3alfa

secretion by osteoblast and osteoprogenitor cells. MM cells also stimulated both CCL20/MIP-3alfa and CCR6 expression by OCs in co-culture. Thereafter we found that CCL20/MIP-3alfa significantly increases both the number of multinucleated $TRAP^+$ OCs and $RANK^+$ OC progenitor cells in presence of RANKL and M-CSF and induces RANKL expression by osteoprogenitor cells. Finally we demonstrated that blocking anti-CCL20/MIP-3alfa and anti-CCR6 antibodies significantly inhibit MM-induced OC formation *in vitro*. These observations were expanded *in vivo* in MM patients. MIP-3a/CCL20 levels have been detected in the BM plasma of MGUS subjects (n=16) and in MM (n=52) patients at the diagnosis in relationship with the presence of bone lesions (osteolytic n=32; non-osteolytic: n°=20). Significant higher MIP-3alfa /CCL20 levels were detected in MM patients vs. MGUS (mean±SD: 51.9±2 *vs.* 21±3 pg/mL; p=0.01) and in MM osteolytic patients vs. non-osteolytic ones (mean±SD: 70.8±5.9 *vs.* 13.8±1.1 pg/mL; p=0.001). Interestingly, no significant differences were observed between MGUS and non-osteolytic MM patients. By immunohystochemistry performed on BM biopsies, we consistently found that MIP-3alfa /CCL20 was over-expressed in osteoblasts in osteolytic MM patients as compared to non-osteolytic ones. In addition we found that OCs showed a strong CCR6 staining in the areas with an increased number of OCs. In conclusion our data indicate that MIP-3alfa /CCL20 its receptor CCR6 are up-regulated in bone microenvironment by MM cells and involved in osteoclast formation and bone lesions in MM patients.

C060

INHIBITION OF OSTEOCLAST APOPTOSIS IN MULTIPLE MYELOMA BONE DISEASE: ROLE OF DCR3

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Multiple myeloma (MM)-bone disease is related to an unbalanced bone turnover with enhanced resorption, related to increased osteoclast (OC) recruitment and activity, and low bone formation. In an *in vitro* osteoclastogenesis model we previously demonstrated that OCs from peripheral blood mononuclear cells (PBMCs) of MM bone disease patients displayed a long life span in culture exclusively when generated in the presence of T cells. In this system the OCs do not undergo apoptosis although high concentration of apoptotic molecules, such as FasL, in the medium. However, why the OCs were protected from FasL-induced apoptosis remained unclear. Since Decoy Receptor 3 (DcR3), member of TNF receptor superfamily, is known to bind and neutralize FasL, in the present study we hypothesized a possible involvement of DcR3 in the inactivation of FasL in our system. We found that T lymphocytes and malignant plasma cells from MM bone disease patients overexpressed DcR3. We also demonstrated the formation of the DcR3/FasL immunocomplex in T- and MM-cell lysates as well as in culture media, suggesting that DcR3/FasL interaction inactivated FasL in the media and OCs were protected from the FasL-induced apoptosis. The viability of MM OCs was significantly reduced in presence of anti-DcR3 neutralizing antibody, indicating that the formation of DcR3/FasL complex could be responsible of the inhibition of FasL-mediated OC apoptosis. We showed that the treatment of MM OCs with anti-DcR3 antibody activated the apoptotic intracellular pathway leading to caspase-8 and caspase-3 cleavage, and DNA fragmentation. Moreover, high DcR3 serum levels were detected in MM bone disease patients respect to controls. Our data demonstrated that T lymphocytes and malignant plasma cells from MM bone disease patients by producing DcR3 protect OCs from apoptosis and contribute to the progression of MM bone disease.

C061

EXPRESSION OF A CHIMERIC T-CELL RECEPTOR SPECIFIC FOR THE CD33 ANTIGEN ON CYTOKINE INDUCED KILLER CELLS RADICALLY IMPROVES THEIR ANTI-LEUKEMIC ACTIVITY

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Acute myeloid leukaemia (AML) is the most common form of leukaemia in adults and accounts for 30% of leukaemia-related deaths in children. Current chemotherapy regimen ensures long-term remission in only 40% of patients, and anti-CD33 monoclonal antibody-based therapy has demonstrated limited efficacy and significant side effects. A CD33-targeting strategy may be improved by the use of CIK cells genetically modified with a CD33-specific CAR. CIK cells are $CD3^+CD56^+$ immune effector cells, easily obtainable *in vitro* from peripheral blood, that display optimal trafficking capacity towards leukaemia-infiltrated tissues and minimal spontaneous killing towards $CD33^+$AML. We efficiently transduced CIK cells with a SFG-retroviral vector carrying an anti-CD33-zeta CAR. In three different donors, average CAR expression was 74% (range, 65-83%). After 4 hours of incubation, anti-CD33-zeta expressing CIK cells displayed potent cytotoxicity against the AML cell line HL-60 (100% of lysis; n=3) and against primary AML cells (>60% of lysis; n=3) at Effector:Target ratio 5:1, whereas non-transduced cells were minimally cytotoxic against those targets (30% and 9% of lysis, respectively; n=3; p=0.05). Similar results were obtained in long-term co-cultures (6 days) on mesenchymal cells with low percentages of CIK cells (E:T ratio 0.01:1). In these assays, CIK cells expressing anti-CD33 receptor had more potent cytotoxicity than non-transduced cells. In fact, average killing of HL-60 cells was 70% (range, 70-98%) versus 20% (range, 6-44%; n=3, each; p=0.05). Moreover, expression of anti-CD33 CAR resulted in a significant increase of cytokine release from transduced CIK cells after 48h stimulation with irradiated HL-60 cells. In 3 different experiments, CIK cells expressing anti-CD33-zeta presented a 12-fold increase of IFN-gamma release (5041 and 428 pg/ml, p=0.05), 13-fold increase of TNF-alfa (1930 and 144 pg/ml, p=0.05), 300-fold increase of TNF-beta (313 and 1 pg/mL, p=0.05), 2-fold increase of IL-8 (4234 and 2316 pg/mL, p=0.05) compared to unmanipulated cells. In conclusion, our *in vitro* results suggest that anti-CD33-zeta transduced CIK cells may represents a promising tool for AML immunotherapy.

C062

HUMAN HERPES VIRUS 6 CHROMOSOMAL INTEGRATION (CIHHV-6) OF RECIPIENT ORIGIN IN ALLOGENEIC BONE MARROW AND SOLID ORGAN TRANSPLANTATION PATIENTS: IMPLICATION FOR LABORATORY DIAGNOSIS AND CLINICAL MANAGEMENT.

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Background. Human herpes virus (HHV) 6, an ubiquitous beta-herpesvirus, may reactivate and cause diseases upon immunosuppressive state. Half of bone marrow (BM) and/or solid organ transplant (SOT) recipients experience HHV-6 viremia; and BM suppression, encephalitis, pneumonitis and hepatitis are being increasingly associated clinical manifestations (Potenza et al New Engl J Med in press). A lesser-recognized form of HHV-6 latency is the integration of the viral genome in a host chromosome (CIHHV-6). This phenomenon, characterized by a high viral copy number in blood or sera, may confound laboratory diagnosis of HHV-6 active infection, but apparently neither affects host health status nor requires clinical intervention. We have studied patients presenting HHV-6 DNAemia during the course of organ transplantation to identify cases of CIHHV-6. *Methods.* 78 allogeneic BMT and 200 SOT were enrolled. HHV-6 loads have been quantified by a commercially available quantitative real time polymerase chain reaction diagnostic kit (Nanogen Advanced Diagnostics, Turin, Italy). *Results.* 40 patients presented HHV-6 viremia (14%). 2 (5%) out of 40 patients, one BMT and SOT respec-

tively, demonstrated high value of HHV-6 DNAemia. The SOT patient presented a mean HHV-6 viral load >3.5 log10 copies/mL in plasma, and >6 log10/mL in blood during the entire clinical course. The alloBMT patient, presented high BM HHV-6 DNAemia (>5 log10 copies/mL) soon after transplantation, reducing to <4 log10 copies/mL with increasing chimerism, while presented fluctuating HHV-6 viremia, from 2 to >4 log10 copies/mL in plasma, unparalleled to white blood cell engraftment and associated with graft versus host disease episodes. Both patients had been heavily treated with antivirals without changes in HHV-6 loads. HHV-6 load in hair follicles in the first patient and oral and gastric cells in the second patient, revealed ≥1 HHV-6 copy/cell, indicating that the virus had been inherited in the germ line and is found in all cell of the body. *Discussion.* These are two cases of CIHHV-6 of recipient origin in transplant patients. Of note the SOT patient is the first report of CIHHV-6 in the SOT setting. CIHHV-6 should be suspected in transplant patients with persistently high viral load. Donors and recipients pretransplantation screening may help to identify CIHHV-6 patients from those with active infection, preventing the former from receiving unnecessary exposure to potentially toxic antiviral drugs.

C063

TRANSPLANTATION FROM HAPLOIDENTICAL MOTHER TO CHILD WITH THALASSEMIA

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We studied the use of the haploidentical mother as the donor of hematopoietic stem cells assuming that the immuno-tollerance established during the pregnancy will help to bypass the HLA disparity and allow the hemopoietic allogeneic reconstitution in the thalassemic recipient of the transplant. We have employed a new preparative regimen for the transplant in 19 thalassemic children aged 3 to 12 years (median age 5 years) using T cell depleted peripheral blood stem cell (PBSCTs) plus bone marrow (BM) stem cells. All patients received hydroxyurea (OHU) 60 mg/kg and azathioprine 3 mg/kg from day -59 until day-11, fludarabine (FLU) 30 mg/m^2 from day -17 to day -11, busulphan (BU) 14 mg/kg starting on day -10, and cyclophosphamide(CY) 200 mg/kg, Thiotepa 10 mg/kg and ATG Fresenius 2,5 mg/kg, followed by a CD34$^+$ T cell depleted (CliniMacs system), granulocyte colony stimulating factor (G-csf) mobilized PBSC from their HLA haploidentical mother. The purity of CD34$^+$ cells after MACS sorting was 98-99%, the average number of transplanted CD34$^+$ cells was 15, 4×10^6/kg and the average number of infused T lymphocytes from BM was 1,8×10^5/Kg.The patients received cyclosporin after transplant for graft versus host disease(GVHD) prophylaxis during the first two months after the bone marrow transplantation. *Results.* Twelve patients are alive disease free with a median follow up of 39 months (range 15-54). Five patients rejected the transplant and are alive with thalassemia. Two patients died, one CMV pneumonia ,one EBV cerebral lymphoma. Seventeen patients are alive. This preliminary study suggest that the transplantation of megadose of haploidentical CD34$^+$ cell from the mother is a realistic therapeutic option for those thalassemic patients without genotipically or phenotipically HLA identical donor. In term of immunological reconstitution after bone marrow transplant Twenty days post transplant, an impaired growth and differentiation capacity of stem/progenitor cells were observed in thalassemia patients, in parallel with an altered homeostasis of T-cells and a reduction of T-cell naïve compartment. We hypothesize that the damage of T cell compartment may be at least partially due to an altered production of new T cells starting from the haematopoietic stem/progenitor cells.

C064

TIME OF PERIPHERAL BLOOD PROGENITOR CELL COLLECTIONS MAY INFLUENCE THE RISK OF SECONDARY MYELODYSPLASTIC SYNDROME/ACUTE LEUKEMIA FOLLOWING INTENSIVE THERAPY AND AUTOGRAFT: A GITIL SURVEY ON 1,347 LYMPHOMA PATIENTS RECEIVING THE HIGH-DOSE SEQUENTIAL PROGRAM

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Introduction. The high-dose sequential (HDS) chemotherapy regimen with peripheral blood progenitor cell (PBPC) autograft is an effective treatment for both non-Hodgkin s (NHL) and Hodgkin s Lymphoma (HL). The occurrence of secondary myelodysplastic syndrome/acute leukemia (sMDS/AL) remains a critical issue, representing a major cause of failure in patients potentially cured after HDS. Aim of the study. To evaluate incidence and risk factors of sMDS/AL in a large series of patients treated with HDS. *Patients and Methods.* Data have been collected on 1,347 patients, who received either the original or the modified HDS regimen in the last two decades at 11 Centers, associated to GITIL (Gruppo Italiano Terapie Innovative nei Linfomi). The series included 234 HL and 1,110 NHL; median age was 46 yrs; 771 were male; 640 (47.5%) patients received HDS front-line; 152 (13%) did not complete the program with autograft. Nearly all patients (97%) were autografted with PBPC (median CD34$^+$ cells: 8×10^6/kg); PBPC were usually collected after hd-cyclophosphamide or after a 2nd round of mobilization, with hd-Ara-C; there were no significant differences in the amount of grafted cells between PBPC of the 1st vs. the 2nd mobilization course. HDS was supplemented with Rituximab in 525 (39%) patients. *Results.* At a median follow-up of 5.5 yrs, 5 and 10 yr Overall Survival projections are, respectively, 62% and 54% for the whole series, 69% and 61% for patients treated at diagnosis. Overall, 46 (3.4%) patients developed s-MDS/AL, with a cumulative incidence of 3.2%, 4.7% and 8.4% at 5, 10 and 20 yrs, respectively. Median time of s-MDS/AL occurrence was 35 months since autograft. In univariate analysis, a few clinical parameters, including age > 45 yrs., male sex, advanced stage, Rituximab administration, autograft with PBPC of 2nd round of mobilization, displayed a variable increase in the incidence of sMDS/AL; however, on competing risk multivariate analysis, only male gender and reinfusion of PBPC of the 2nd mobilization course were associated with sMDS/AL occurrence (SDHR: 2.93, p=0.007 for male gender; 2.54, p=0.004 for graft with PBPC collected at the 2nd round). *Conclusions.* i. the incidence of sMDS/AL in HDS-treated patients is analogous to that reported in other recent surveys on lymphoma patients following autograft, with a higher risk recorded among males; ii. the quality of CD34^{+ve} cells employed for autograft may be critical for the post-graft development of sMDS/AL.

POSTERS

Stem Cells and Growth Factors

P001

C-MYB SILENCING IN HUMAN HEMATOPOIETIC STEM/PROGENITOR CELLS INDUCES THE MACROPHAGE AND MEGAKARYOCYTE DIFFERENTIATION

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The c-Myb transcription factor is highly expressed in immature hematopoietic cells and down-regulated during differentiation. To define the role of c-Myb in human hematopoietic lineage commitment, we studied the effects of its silencing during the commitment of human primary CD34$^+$ hematopoietic stem/progenitor cells and CD14$^-$ myeloblasts. In CD34$^+$ cells c-Myb silencing determines a G0/G1 phase cell cycle arrest which strongly decreases the clonogenic efficiency, togheter with a reduction of erythroid colonies (BFU-E, CFU-E) and an increase of the macrophage (CFU-M) and megakaryocyte (CFU-MK) colonies. Morphological and flow cytometry data support the macrophage and megakaryocyte commitment of c-Myb-silenced CD34$^+$ cells. Consistently, in CD14$^-$ myeloblasts, which maintain a monocyte-granulocyte differentiation ability, c-Myb silencing determines the G0/G1 arrest and the differentiation to macrophages. Taken together our data indicate that c-Myb is essential for the proliferation of hematopoietic stem/progenitor cells and myeloblasts and for the commitment along the erythroid and granulocyte lineages but not for the macrophage and megakaryocyte differentiation. These effects cannot be merely ascribed to the arrest in G1 phase, as the silencing of D cyclins, which directly act only on the G1/S phase transition and not on the commitment and differentiation pathways, recapitulates the cell cycle arrest obtained by the silencing of c-Myb but it does not affect significantly the commitment of CD34$^+$ cells. However, gene expression profiling and computational analysis of promoters identify some potential c-Myb targets which can account for the biological effects of c-Myb silencing on CD34$^+$ stem/progenitor cells lineage commitment. Indeed, preliminary ChIP data demonstrate the direct binding of c-Myb to the promoters of the LMO2 and MAFB genes.

P002

ADULT BONE MARROW-DERIVED MESENCHYMAL STEM CELLS EXPRESS A FUNCTIONAL P2X7 RECEPTOR WHOSE STIMULATION INDUCES PLASMA MEMBRANE PERMEABILIZATION BUT NOT CELL DEATH

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Human adult bone marrow-derived mesenchymal stem cells (ahMSC) are multipotent stem cells endowed with the ability to differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes, adipocytes, beta-pancreatic cells etc. Particular interest has been focused on this cell type for clinical uses in tissue engineering and regenerative medicine, therefore it would be desirable to develop new efficient methods of isolation, expansion, and site-directed delivery. Extracellular nucleotides represent a new class of extracellular messengers binding to plasma membrane receptors named P2 receptors. Very little is known on the role of extracellular nucleotides in stem cells biology. Here we demonstrate that ahMSC expressed the mRNAs for P2X1, P2X4, P2X7, P2Y1, subtypes. Bone marrow-derived ahMSC underwent plasma membrane permeabilization in the presence of extracellular ATP or the P2X7 agonist BzATP. Peculiarly, although ahMSC expressed a functional P2X7 subtype, they where quite refractory to ATP- or BzATP-induced cell death as demonstrated by the scarce release of the cytosolic marker lactate dehydrogenase (LDH). A prolonged exposure to high ATP concentrations did not induce relevant changes in ahMSC morphology and UTP was completely ineffective. Moreover, although ATP induced a decrease in plasma membrane potential, mitochondria of ahMSC were not swollen or fragmented even after hours in the presence of high extracellular ATP concentrations. These findings show that although ahMSC express functional P2 receptors, they are somehow able to control/avoid nucleotide-mediated cytotoxic effects. It is therefore worthy to further investigate this issue in view of its potential use in clinical applications of ahMSC.

P003

EXTRACELLULAR NUCLEOTIDES MODULATE HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELL FUNCTIONS

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Human bone marrow mesenchymal stem cells (hMSCs) have the potential to originate different cell types and this fact is of great importance for regeneration of damaged tissues such as for heart muscle after infarction or muscle cells and cartilages upon traumatic events. Particular interest has been focused in recent years on the role of extracellular nucleotides in modulating cell functions, but little is known about their effects on hMSCs. Extracellular nucleotides represent a recently uncovered class of extracellular messengers. They bind to specific plasma membrane receptors (P2 receptors) that are widely expressed and modulate responses as different as proliferation, differentiation, migration or cell death. In this study, we assessed whether receptors for extracellular nucleotides were expressed and functional in hMSCs. Western blot analyses showed expression of the P2X1, P2X4, P2X7 and P2Y1 subtypes. Of notice the fact that P2X4 protein was very abundant in hMSCs. P2 receptors stimulation by ATP or UTP induced intracellular Ca2+ concentration changes in hMSCs. The P2X7 receptor ligand 2',3'-(4-benzoyl)benzoyl-ATP was less potent than ATP or UTP in inducing the Ca2+ increase. The covalent P2X receptor blocker oxidized ATP (oATP) or the P2X7 specific antagonist KN-62 reduced Ca2+ changes induced by BzATP, particularly the plateau phase. At the functional level, incubation with extracellular nucleotides did not modify hMSCs proliferation, phenotype and immunomodulation properties. Whereas both ATP and UTP exerted a chemotactic effect on hMSCs similar to that of the chemokine CXCL12. Our data show that extracellular nucleotides modulate hMSCs functions and point to a role of these mediators in hMSCs biology.

P004

A STUDY OF THE CROSSTALK BETWEEN CD30 AND CXCR4

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Background. CD30, a receptor belonging to the TNFR superfamily, and the chemokine receptor CXCR4 are often co-expressed in several biologically relevant conditions, such as lymphoid adaptive response, polarization towards Th2 function, and thymocyte maturation. Also a number of human-derived hematological cell lines tipically co-express CD30 and CXCR4, representing a useful model to study the crosstalk between these receptors. Methods. Thus, we studied a panel of CD30$^+$ CXCR4$^+$ cell lines including L540, Jurkat, L428, Karpas-299 and Molt-3. The effect of 100 ng/mL CXCL12 (SDF-1alfa) was evaluated after pre-stimulation of CD30 with 50 ug/mL HRS4 agonistic antibody for different times (1, 6 and 24 hours). The CXCR4 internalization and chemotactic activity induced by these stimuli were measured by flow cytometry methods. CXCR4 mRNA transcripts were evaluated at the same time points by Northern blot and RT-PCR. Cell proliferation was measured by CarboxyFluorescein Diacetate Succinimidyl Ester (CFSE) labeling. Results. After 1 hour of stimulation with CXCL12, CXCR4 mean fluorescence intensity (MFI) decreased in all the studied cell lines, due to receptor internalization. When pre-incubated 1 to 6 hours with HRS4, L540 and Karpas-299 cell lines showed a clear inhibition of CXCL12-dependent internalization of CXCR4 (Table 1), whereas Jurkat, L428 and Molt-3 cell lines retained a normal pattern of CXCR4 internalization. After 6 to 12 hours of pre-incubation with HRS4, CXCR4 mRNA transcripts and

CXCR4 surface density were increased in L540 and Karpas-299 cells (p<0.01), whereas in Jurkat, L428 and Molt-3 cell lines no differences were observed. Thus, HRS4 pre-stimulation induced CXCR4 neosynthesis in L540 and Karpas-299 cell lines. Pre-stimulation with HRS4 regulated also CXCL12-dependent chemotactic activity in L540 and Karpas-299 cells. Chemotactic index decreased from 8.5±1.4 and 28±4.2 in basal conditions, to 1.2±0.2 and 11.1±1.9 after 1 hour of pre-stimulation with HRS4 (p<0.01). After 24 hours of HRS4 pre-stimulation, CXCL12-dependent chemotaxis was fully restored, in parallel with the increased expression of CXCR4. Jurkat, L428 and Molt-3 cell lines chemotaxis was unaffected by HRS4 pre-stimulation. We found that both Karpas-299 and L540 were $CD4^+$ $CD25^+$ $Foxp3^+$, which assigned the phenotype of regulatory T lymphocytes (Treg) to these cell lines. In co-colture experiments, they both inhibited the proliferative activity of normal T lymphocytes activated via anti-CD3/CD28. Conclusions. The cross-talk between CD30 and CXCR4 described above seems to be restricted to cells displaying Treg activity, suggesting that CD30 determines a different modulation of chemotactic response in Treg as opposed to non-Treg cells.

Table 1. CXCR4 mean fluorescence intensity (MFI) of the cell lines under different conditions.

Cell line	*Basal*	*+ CXCL12*	*+HRS4*	*+HRS4 +CXCL12*	*p**
L540	358,06	253,08	396,87	369,82	ns
Karpas-299	331,49	218,13	324,87	336,16	ns
Molt3	569,34	413,51	572,26	397,27	<0.01
Jurkat	458,12	283,49	394,6	259,01	<0.01
L428	514,16	237,23	508,7	284,75	<0.01

**p was calculated as difference between CXCR4 MFI after preincubation with HRS4 ±CXCL12.*

P005

ISOLATION AND CHARACTERIZATION OF L-NGFR$^+$ MESENCHYMAL STEM CELLS FROM ADIPOSE TISSUE AND BONE MARROW: A COMPARISON BETWEEN THE TWO SOURCES

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Adult stem cells hold great promise for cell-based tissue engineering for repair and regeneration of tissues damaged by disease or injury. Mesenchymal stem cells (MSCs) are a population of multipotent cells that can proliferate and differentiate into multiple mesodermal tissues such as bone, cartilage, muscle, ligament, tendon and adipose. We have previously reported that monoclonal antibodies (MoAbs) to the low-affinity nerve growth factor receptor (L-NGFR – CD271) stain primitive MSCs with high specificity and purity in adult bone marrow (BM), defining a subset with a high proliferative potential and clonogenic efficiency, and the ability to differentiate into multiple mesodermal tissues. In the recent years, numerous studies have shown that adipose tissue (AT) is an alternative source of adult multipotent stem cells, able to differentiate into several mesodermal lineages. Their use is not complicated by ethical controversies and they are easily accessible in large amounts under local anesthesia by the minimally invasive procedure of liposuction. The objective of this study was to characterize and compare AT L-NGFR$^+$ cells to those isolated from BM. BM and AT L-NGFR$^+$ cells were analyzed by flow cytometry immediately after immunoseparation: the mean percentage of L-NGFR$^+$ cells was 1.61%±0.65 in BM *vs.* 5.24%±6.8 in AT. Both populations expressed surface markers associated with a primitive phenotype: we observed a significant decrease in CD45, CD133 and CD105 coexpression comparing BM to AT (69.3%±21 *vs.* 2.4%±1.8, 3.5%±5.4 *vs.* 0.4%±0.4, 79.7%±13.8 *vs.* 40%±45, respectively), while CD34 coexpression markedly increased (22.6%±6.6 *vs.* 76.8%±12.6) and was maintained with a mild progressive decrease during culture. CD117, KDR, P1H12 were coexpressed at similar levels. The clonogenic efficiency of BM L-NGFR$^+$ cells was higher immediately after separation, but rapidly decreased during culture, while AT L-NGFR$^+$ cells showed lower but stable clonogenic activity. When cultured without the addition of specific growth factors, AT L-NGFR$^+$ cells showed a 2-3 log greater expansion ability and maintained the capacity to differentiate towards adipogenic, osteogenic and chondrogenic lineages throughout the time of culture. In addition, BM L-NGFR$^+$ cells proliferation ability exhausted within 8-10 weeks, while AT L-NGFR$^+$ cells were able to grow up to 20-30 weeks. These data suggest that AT L-NGFR$^+$ cells can be an alternative and promising source of pluripotent MSCs for tissue engineering.

P006

PERITRANSPLANT ADMINISTRATION OF THE ORAL DIPEPTIDYL DIPEPTIDASE IV INHIBITOR SITAGLIPTIN MODIFIES SERUM SDF-1ALPHA LEVELS

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Hematopoietic stem cell (HSC) engraftment into recipient bone marrow is mainly driven by gradients of the chemokine CXCL12 (also known as stromal cell-derived factor 1). Alternative splicing generates 2 isoforms (alpha and beta) differing by 4 amino acid residues in the carboxyterminus. No drug currently exists to modulate this gradient. CXCL12 is inactivated when the 2 amino-terminus amino acids are cleaved by dipeptidyl peptidase IV (DPP-IV). Recently, DPP-IV inhibitors (gliptins) have been clinically approved as oral antidiabetes agents. We tested whether sitagliptin was safe and effective at modifying serum SDF-1alpha levels in a homogeneous cohort of multiple myeloma patients receiving high-dose melphalan and HSC transplantation, and tested whether the drug could improve engraftment and reduce transfusion requirements. 8 consecutive patients (6 males and 2 females; median age 59 years) with multiple myeloma receiving high-dose melphalan (range: 100 to 200 mg/m^2 of body surface area) on day -2 and HSC transplantation (mean dose : 4.1 millions $CD34^+$ HSCs per kg of body weight) on day 0 were treated with oral sitagliptin (Januvia, Merck & Co., Inc) 100 mg b.i.d. since day -1 until day +2. All patients also received pegfilgrastim on day +1, platelet transfusions when lower than 20000/microliter (median : 1 concentrate) and packed red blood cell units when hemoglobin was lower than 8 g/deciliter (median : 1 concentrate). Peripheral blood samples were collected on day -2, 0, +3, +6 and +10. Serum SDF-1alpha levels were measured with the Human SDF-1alpha Quantikine Colorimetric Sandwich ELISA (R&D System, Minneapolis, MN, USA). The drug was well tolerated without any significant adverse effect. In comparison with a historical cohort of first and seconds autologous HSC transplantation (n=31), patients receiving sitagliptin exhibited significatively lower serum SDF-1alpha levels during the days of treatment, returning to baseline levels on day +10 (as depicted in Figure 1). We demonstrated for the first time that DPP-IV inhibitors have the potential to modify serum SDF-1alpha levels. Apart from autologous HSC transplantation these drugs could potentially be added to the armamentarium against allogeneic graft failure.

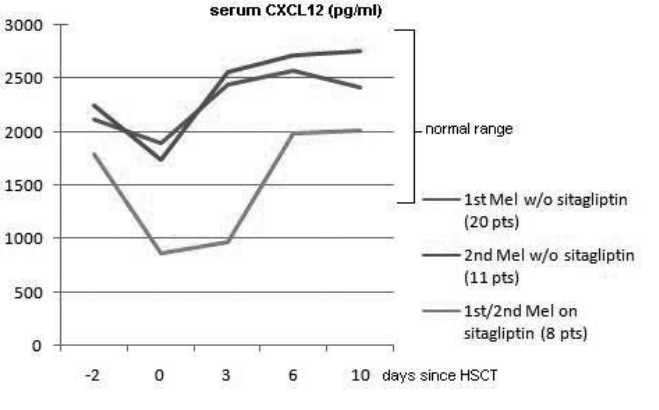

Figure 1.

P007

PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF CD4+CD25+ REGULATORY T CELLS IN CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA

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$CD4^+CD25^+$ 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 chronic immune thrombocytopenic purpura (ITP), an immune disorder in which increased platelet clearance is caused by antiplatelet autoantibodies, has not yet been clarified. Therefore, the aim of this study was to explore the number and the function of Tregs in ITP patients. Using two novel markers ($FoxP3^+$ and $CD127^-$), we evaluated by flow cytometry the frequency and the absolute number of Tregs in peripheral blood of 20 patients with active ITP and 20 healthy subjects. In addition, to investigate the suppressive activity of Treg cells, proliferation assays were performed in which $CD4^+CD25^-$ T cells were cultured in absence or presence of $CD4^+CD25^+$ T cells at the ratio 1:0, 0:1, 1:1. Patients, 5 males and 10 females, were at diagnosis (8 cases) or at least 3 months from therapy (7 cases). The median platelet number at the time of the study was 49×10^9/L (range 8-98). The absolute number and the percentage of $CD4^+CD25^{high}Foxp3^+$ T cell population was significantly decreased in ITP patients (5.46±5.39/microL; 73.83±17.38%) when compared to healthy subjects (11.62±6.98/microL; 89.61–10.60%; $p<0.01$). Consistently, ITP patients had significantly lower absolute number of $CD4^+CD25^{high}CD127^{low/neg}$ T cells (50.99±27.29/microL) than that of healthy controls (80.49±37.71/microL; $p<0.02$). On this basis, we also evaluated the suppressive capacity of $CD4^+CD25^+$ T cells in healthy subjects and ITP patients. We demonstrated that the $CD4^+CD25^+$ T cells of ITP patients and healthy subjects show the capacity to inhibit the proliferation of the $CD4^+CD25^-$ T cells. Taken together, these results suggest that in ITP patients Tregs abnormalities may contribute the pathogenesis of the disease. *Supported in part by BolognAil.*

P008

SINGLE DOSE OF PEGYLATED RECOMBINANT FILGRASTIM (PEGFILGRASTIM) AFTER CONSOLIDATION CHEMOTHERAPY FOR ACUTE MYELOID LEUKEMIA IN FIRST COMPLETE REMISSION

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Introduction. Clinical trials are currently expanding the clinical experience with Pegfilgrastim in a variety of solid tumors and hematologic malignancies. Previous reports showed that Pegfilgrastim was as effective as daily Filgrastim after induction chemotherapy in AML in order to reduce time to recovery from granulocytopenia. No data are available about the role of Pegfilgrastim after consolidation chemotherapy in AML in first complete remission (CR). *Patients and methods.* A total of 32 consecutive AML pts (17M/15W) in first CR (median age 50 yrs, range 25-69) received Pegfilgrastim (6 mg single dose, subcutaneously) 24 hours after completion of consolidation chemotherapy that consisted of cytosine arabinoside 2 g/m^2 for 6 days plus idarubicin 12 mg/m^2 day 1-3 (26/32), or cytosine arabinoside alone 3 g/m^2 twice day for 4 days (6/32). This is a setting of pts with an expected prolonged and severe neutropenia and all of 32 cases have previously received the same intensive induction therapy with fludarabine containing regimen (FLAI). *Results.* All 32 pts, as expected, experienced grade IV WHO neutropenia after consolidation chemotherapy. Median time to PMN recovery (first of 2 consecutive days with PMN >$0,5\times10^9$/L and with PMN >1×10^9/L) from Pegfilgrastim was 15 and 17 days, respectively. The mean peak value of PMN was $6,9\pm6,3\times10^9$/L and occurred after a median of 22 (range 10-43) days from Pegfilgrastim injection. Sixteen (50%) pts experienced infectious complications during the aplastic phase (3 pneumonias, 10 bacteremias, 1 cystitis and 1 skin abscess) but no deaths infection related were reported. Pegfilgrastim was well tolerated and only 1/32 pts required pain-control medications (osteoalgia). In 34% of cases, without PMN >1×10^9/L after 15 days from Pegfilgrastim, Filgrastim (mean 3 fl/pts) were administered in order to accelerate PMN recovery. Nine of 32 pts (28%) underwent leukapheresis procedures when the $CD34^+$ cell count was more than 10 micronL (mean $CD34^+$ cell count at collection was 105±128 micronL). The mean number of harwested $CD34^+$ cells was $5\pm3,8\times10^6$/Kg after a mean time from Pegfilgrastim of 15±6 days. *Conclusions.* Our experience confirms that Pegfilgrastim: 1) was well tolerated after consolidation chemotherapy in AML in first CR; 2) represent a cost-effective alternative to long–term conventional Filgrastim to overcome severe and prolonged granulocytopenia after consolidation CHT in AML; 3) could permit simplification of PBSC mobilization procedures and harvest in AML. In our experience only 28% of pts obtained $CD34^+$ cells mobilization and harvest. However this is not a bad result taking into account the underling disease (AML) and the previous induction with fludarabine based regimen that is well know to severly impair PBSC mobilization and collection.

P009

CYTOSINE ARABINOSIDE EFFECTIVELY MOBILIZES PBSC IN A POOR MOBILIZED MULTIPLE MYELOMA PATIENTS

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Double autologous stem cell transplantation is performed in an increasing number of Myeloma Multiple (MM) patients (pts). The number of Peripheral Blood Stem Cells (PBSC) necessary for double transplant is high , a small number of patients failed first mobilization of CD34+ PBSC. There are contradictory studies about the effectiveness of second mobilization strategies in these patients. From February 2006 to March 2008 we treated 8 poor-mobilization MM pts, (4 IgG, 3 Micromulecolar, 1 IgA , 5 stage III A Durie and Salmon , 1 III B, 1 II ,1 I A), median age 59,37 years (range 50-66), 6 male, 2 female, the median follow up to diagnosis was 1,75 years (range 1-3 years).The pts underwent previously to 1 lines (6 pts) or 2 lines (2 pts) of chemotherapy; (1 DAV and Desametasone,1 DAV and Talidomide + Desametasone, 3 Talidomide +Desametasone, 2 DAV only , 1 DEXA only). The first treatment of mobilization was Cyclophosmamide 4 gr/mq following G-CSF 10 mcg/Kg. After CTX mobilization a median of 23,28 cell/mcl was count of peripheral $CD34^+$ cells (range 11-46) not enough for double autologous transplantation. These Patients failed mobilization or not achieving the target of 2.5×10^6 $CD34^+$ cells/kg underwent a second attempt using intermediate-dose (ID) Ara-C, 800 mg/mq every 12 h for six doses + G-CSF. The target yield was > 5.0×10^6 $CD34^+$ cells/kg. All pts mobilized peripheral stem cells. A median of 510 cells/mcl was count of peripheral $CD34^+$ cells (range 228-797). A median of $19,87\times10^6$ $CD34^+$ cells/kg was collected per pts (range 12-28). The median time of the first collection was 14,9 days (range 11-21).The median number of apheresis to obtain the target was 1,12 (range 1-2) The status at time of mobilization was: 6 pts in partial remission (PR), 1 pts in near complete remission (nCR) 1 in progression. Only 3 pts received packed red blood cells(2 for pts),while platelet support was needed in 4 pts,. 4 pts experienced mucositis W.H.O. grade 2, 2 patients had fever W.H.O. 2, In conclusion, stem cells collection with intermediate-dose (ID) Ara-C is feasible in MM pts failed previously first line mobilization. The toxicity was manageable The mechanisms of ARA-C on stem cell harvest must be further investigated

P010

PEGFILGRASTIM COMPARED WITH UNCONJUGATED G-CSF PLUS CHEMOTHERAPY AS MOBILIZATION OF PERIPHERAL BLOOD STEM CELLS IN LYMPHOMA AND MYELOMA PATIENTS

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High-dose chemotherapy followed by autologous blood stem cell transplantation are currently used in myeloma and lymphoma patients. Few data have been reported in literature on the use of Pegfilgrastim to mobilize CD34+ peripheral stem cells after chemotherapy in these patients. Aims. We undertook a randomized study to evaluate the efficacy of pegfilgrastim plus chemotherapy vs. non pegylated G-CSF in term to mobilize peripheral blood CD34+ cells and to reduce the poor mobilizer phenomenon in patients with lymphoprolipherative disorders. *Methods.* From March 2005 to April 2008, 79 patients (46 non Hodgkin Lymphoma, 11 Hodgkin Lymphoma, 22 Multiple Myeloma) were randomized to receive 6 mg pegfilgrastim on day 4 (median, range 2-7) or daily doses of G-CSF (10 micrograms/Kilogram/day) from day 4 (median, range 2-5) after mobilization chemotherapy for a total of 95 harvesting procedures. Table 1 shows the clinical characteristics of enrolled patients (39 with pegfilgrastim and 56 with G-CSF). No significant differences concerning clinical characteristics are tested between the two cohorts of patients.

Table 1. Characteristics of enrolled patients.

	All	*Pegylated-GCSF*	*G-CSF*	*p*
Number of procedures	95	39 (41%)	56 (59%)	
Age, median (range) years	53 (15-77)	55 (16-77)	49 (15-76)	0.19
Gender, n (%)				
Male	54 (57)	22 (41)	32 (59)	0,7
Female	41 (47)	17 (41)	24 (59)	
Diagnosis, n (%)				
Non Hodgkin Lymphoma	55 (58)	20 (36)	35 (64)	0.47
Aggressive	39			
Follicular	16			
Hodgkin Lymphoma	12 (12)	5	7	
Multiple Myeloma	28 (29)	14	14	
BOM involvment at diagnosis, n (%)	31 (46)	14 (45)	17 (55)	0.4
IPI > 2, n (%)	22 (76)	5	17	1.15
FLIP > 3, n (%)	8 (57)	44		
Number of previous chemoterapy regimens at mobilization,				
median (range)	2 (1-8)	2 (1-6)	2 (1-8)	0.16
> 2	18 (19)	10 (55)	8 (45)	
Mobilization regimens, n (%)				
High Dose Ara-C	55 (58)	21	34	0.35
High Dose Cyclofosphamide	20 (21)	9	11	
Other	20 (21)	9	11	
Status disease				
Responsive	79 (83)	32	47	0.80

Results. A median of 2 apheresis (range 1-3) was performed (Table 2). No difference was observed regarding the day of CD34 peak and maximal CD34 count in the peripheral blood. The total number of collected CD34+ cell/kilogram was similar in the two cohorts: 5.4×10^6 (range 1.06-24.88) in the pegfilgrastim and 6.9×10^6 (range 1.09-32.8) in the G-CSF (p=0.1). Mobilization failure occurred in 19 (20%) patients (6 in pegfilgrastim and 13 in G-CSF, p<0.3). Twenty-four patients (25%) reached <2 CD34+$\times10^6$ (8 and 16 in the pegfilgrastim and G-CSF, p<0.39). Forty-six patients underwent ASCT. Patients mobilized by pegfilgrastim received lower number of CD34+ cells/Kg: 3.9×10^6 (range 1.04-7.3) in comparison to G-CSF group: 5.98×10^6 (range 0.88-21.3); p=0.08. Despite the lower number of CD34+ cells transplanted there was no significant difference regard to the time to haematological engraftment and immunological reconstitution at day + 90 (Table 3). Thirty-three patients (72%) experienced a febrile neutropenia (68% in the pegfilgrastim and 78% in the G-CSF group, not significant p). The early death rate before day 90 was 26% (5/7 cases of TRM and 2 for progression disease). The 90 day- death rates were similar in the two groups. The median follow-up of transplanted patients was 216 days (36-1050). *Conclusions*. In summary, a single dose of pegfilgrastim after chemotherapy is hightly effective as conventional daily G-CSF to mobilize a sufficient number of CD34+ cells useful for a stable engraftment and immunological reconstitution after ASCT. Pegfilgrastim seems to be associated with a trend in reduction of mobilization failure and number of poor mobilizers. Further studies, including double dose of Pegfilgrastim, are warranted to address this issue.

Table 2. Leukapheresis data.

	N (%)			
	All 95	*Pegylated-GCSF* 39 (41%)	*G-CSF* 56 (59%)	*p*
Median apheresis procedures	2 (1-3)	2 (1-3)	2 (1-3)	0.36
>2	9	4	5	
Median day to 1st apheresis	13 (9-24)	13 (10-16)	13 (9-24)	0.67
Maximal peripheral blood Cd34+ count/microlitre	32,2 (1,13-492,5)	24 (3.6-492.5)	39.16 (1,13-367.2)	0.2
>32,2	36 (49%)	13 (39%)	23 (56%)	
WBC count at 1st apheresis, median (range)	4790 (1000-34000)	4790 (1000-19700)	4315(1000-34000)	0.9
Median CD34+x10^6/kg	6,35 (1.06-32.84)	5,4 (1.06-24.88)	6.9 (1.09-32.8)	0.1
Poor mobilizer (<2CD34+x10^6/kg)	24 (25%)	8 (20%)	16 (29%)	0.39
Mobilization failure	19 (20%)	6 (15%)	13 (23%)	0.3

Table 3. Haemapoietic recovery and recovery of CD3+, CD4+, CD8+ and CD56+ cells.

	Median time in Days (range)			
	All 79 patients	*Pegylated-GCSF* 30	*G-CSF* 49	*p*
Neutrophil reconstitution > 0.5x10^9/litre	10 (7-47)	15 (10-43)	11 (7-47)	n.s.
Platelets reconstitution > 20x10^9/litre	13 (8-55)	10 (9-21)	13 (8-55)	

P011

DETERMINATION OF PERIPHERAL BLOOD CYTOKINES AND BONE MARROW FLOW CYTOMETRY ANALYSIS IN PATIENTS WITH MYELODYSPLASTIC SYNDROME TREATED WITH AZACITIDINE

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Background. Myelodysplastic syndromes (MDS) are characterized by multistep pathogenesis: clonal mutation of hemopoietic stem cells (identified by phenotypic abnormalities) and ineffective hematopoiesis with peripheral blood cytopenias mainly caused by apoptotic and pro-inflammatory cytokines (TNF-alpha,TNF-beta,IL 6) on microenvironment derived signals. Opposite data have been described about increased TNF alpha levels in the marrow microenvironment and peripheral blood of MDS patients: some authors consider it an indicator of poor prognosis and early evolution in acute luekemia, others found elevated TNF alpha in low-risk MDS with enhanced erythroid response. *Aim.* The aim of this study is to explore the biological and immunoregulatory effect on peripheral blood cytokines and bone marrow cells phenotype in patients with MDS treated with azacitidine. *Methods.* Monthly cytokine expression (IL2, IL2R, TNFalpha, TNF beta, sTFR, IL6, IL6R) by ELISA and bone marrow flow cytometry before and after the end of therapy (CD13, CD33, CD34, CD14, CD61, CD117, CD56, glycophorin) were analized in 9 patients with MDS treated with 5 azacitidine 75 mg/m^2 s.c. for 7 days every 4 weeks for a total of 6 cycles. Patients had a median age of 66 years(range 50-83) and were rappresented by following WHO subtype (1 AR, 2RCMD, 1RAEB1, 4 RAEB2, 1 AML with previous MDS). The mean International Prognostic Score System was 1.5 and ranged from 0 to 3.5.The overall response rate was 6/9(66%): RC (3/9-33%), RP (3/9-33%),NR (3/9-33%). *Results.*In all patients IL2 before treatment was elevated.There was no statistical difference between patients with IPSS low grade and high grade for elevated level of IL2, IL2R, TNFalpha, TNF beta, sTFR, IL6, IL6R (p<0.02). We describe for all patients with overall response a shortly increase of IL2, TNFalpha, TNF beta, IL6 after first cycle of azacytidine with following decrease during the others cycles. Flow cytometry data were translated into a numerical bone marrow blasts count and there were coincidence . Beside in 3 patients flow cytometry identified aberrancies in the myelomonocytic lineage not otherwise determined by cytomorphology (CD117 on granulocytes, and CD56 on monocytes). *Conclusions.* The role of the microenvironment in the pathophysiology and progression of MDS has remained controversial, may be useful in conducting further experiences on a larger number of patients than us, to confirm this biological model.

Molecular Biology

P012

MDM2 INHIBITION SYNERGISTICALLY ENHANCES CYTOTOXICITY OF MEK1 INHIBITOR AND ARSENIC TRIOXIDE (ATO) IN AML BLASTS THAT RETAIN A FUNCTIONAL P53 PATHWAY

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TP53 mutations are quite rare in AML (5-10%) and MDM2 (murine double minute 2), its principal negative regulator, has been found to be frequently overexpressed in AML, a process that can actively enhance tumorigenic potential and resistance to apoptosis. We recently reported that PD184352 (PD) (Pfizer, Ann Arbor, MI), a highly selective inhibitor of MEK1 phosphorylation and activation, strikingly enhances ATO-mediated apoptosis in NB4, K562 cell lines and in primary AML via p73-p53AIP1 pathway activation. The aim of this study was to investigate whether Nutlin-3, a potent and selective small-molecule MDM2 antagonist, can potentiate the apoptotic effect of the combination PD/ATO in AML cells that retain a wild type p53. We first analyzed the pharmacologic interaction between Nutlin-3, PD and ATO using a fixed-ratio experimental design in OCI-AML-3 and MOLM-13 cell lines that have wild-type p53 and high levels of MDM2. In both cell lines we found that Nutlin-3/PD/ATO showed cytotoxic synergism stronger than PD/ATO indicating that the inhibition of the p53-MDM2 interaction can positively influence the proapoptotic efficacy of PD/ATO-treated cells. We also found that Nutlin-3 significantly (p<0.01) enhanced the PD/ATO-induced apoptosis in fifty percent of the primary AML blasts samples ex vivo analyzed (5 out of 10). In order to investigate the molecular effectors involved in Nutlin-3/PD/ATO or PD/ATO-induced apoptosis we first studied the kinetics of p53 and p73 in OCI-AML-3. In absence of Nutlin-3, ATO even at high doses, did not promote a p53 accumulation whereas modulated the expression of the p73 gene by inducing both the proapoptotic and antiproliferative TAp73 and the antiapoptotic and proproliferative DNp73 isoforms, thereby failing to elevate the TA/DNp73 ratio. Conversely, treatment with PD reduced the level of DNp73 and blunted the ATO-mediated up-regulation of DNp73 thus causing an increase in the TA/DNp73 ratio of PD/ATO-treated cells. In presence of Nutlin-3, p53 accumulated and enhanced the loss of mitochondrial membrane potential occurred in PD/ATO treatment. These findings suggest that the proapoptotic p73 pathway, involved in PD/ATO efficacy, can be potentiated by the rescue of p53 in AML cells that possess a wild type p53. In conclusion, these results suggest that MDM2 antagonists, in combination with PD and ATO, could be a therapeutic strategy that warrants attention in AML.

P013

GENE EXPRESSION DOWNREGULATION BY HAPLOINSUFFICIENCY MECHANISM IN CHRONIC MYELOID LEUKEMIA WITH DELETIONS ON DER(9)

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Background. Genomic deletions flanking the breakpoint on der(9)t(9;22) occur in 10-15% of patients with chronic myeloid leukemia (CML). The deletions on der(9) are associated with a poor prognosis on IFN-alpha therapy whereas controversal data are available about their influence on the response to imatinib. The molecular mechanisms responsible for this unfavourable prognosis are still unclear. *Methods.* 334 CML patients in chronic phase were analyzed by FISH experiments with probes specific for ABL and BCR genes. Sixty (18%) out of 334 cases showed genomic deletions on der(9) chromosome; a detailed characterization of deletions extension was performed by using specific BAC contigs. Because of RNA sample availability, gene expression studies were performed in 30 out of 60 CML patients bearing der(9) deletions and in a pool of 10 CML cases without microdeletions. A total number of 47 genes with known functions were found located inside the 9 and 22 chromosome regions most frequently deleted in CML patients. Among all selected genes, 37 resulted to be homogeneously expressed in normal

bone marrow samples and were analyzed by real-time polymerase chain reaction (qRT-PCR) experiments. A total number of 28 genes was validated by efficient primers pairs; their expression was evaluated in 30 CML cases bearing der(9) deletions and compared to a pool of cDNA samples derived from 10 CML patients without sequence deletions (calibrator). *Results.* All the 28 analyzed genes were found down-regualted with respect to CML cases without deletions. However, the difference was statistically significant only for six: protein kinase PKN3 (PKN3, p=0.003), SH3-domain GRB2-like endophilin B2 (SH3GLB2, p=0.0018), protein phosphatase 2A regulatory subunit B' (PPP2R4, p=0.007), ankyrin repeat and SOCS box-containing 6 isoform (ASB6, p=0.002), ubiquitin specific protease 20 (USP20, p=0.010), and torsin family 1 member B (TOR1B, p=0.009). The expression levels of the downregulated genes were 0.096, 0.222, 0.223, 0.198, 0.222, and 0.293 for PKN3, SH3GLB2, PPP2R4, ASB6, USP20 and TOR1B, respectively. All 6 downregulated genes are located on chromosome 9 sequences, centromeric to the ABL gene, and are implicated in crucial cellular pathways. *Conclusions.* We have showed for the first time an expression downregulation of genes located on der(9) chromosome in CML patients bearing genomic microdeletions. These findings support the haploinsufficiency hypothesis, suggesting that in these cases one allele is always not sufficient to ensure an adequate gene expression dosage.

P014

SEARCHING FOR NEW NUP98 TRANSLOCATION PARTNERS AND THEIR LEUKEMOGENIC ROLE

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Background. Recurrent leukemogenic events in myeloid and T lymphoid malignancies include translocations involving the 5' region of the nucleoporin gene, NUP98, on chromosome 11p15.5. Homeobox (HD) and non-homeobox (NHD) proteins are among the twenty-two partners identified so far. The NUP98/HOXA9 fusion gene provides aberrant self-renewal capacity, blocks myeloid differentiation *in vitro* and induced an AML-like disease *in vivo* after long latency. *Aim.* To identify new NUP98 recombinations in hematological malignancies with 11p15 abnormalities and characterise fusion partners molecularly and functionally. *Methods.* Two cases of acute myeloid leukaemia (AML) with 11p15.5 breakpoints were studied. Patient 1, 28 years old, had 46,XY,t(3;11) (q12;p15). Patient 2, 59 years old, had 46,XY,t(10;11) (q23;p15). Metaphase FISH and RACE-PCR experiments sought the NUP98 partners in both. Cell cultures and murine model experiments evaluated sub-cellular localization and transforming activity in patient 2. Results. Metaphase FISH and 3'RACE experiments identified two new fusion transcripts: NUP98-LOC348801 (patient 1) and NUP98-HHEX (patient 2). Nuclear localization pattern of EGFP-NUP98-HHEX fusion was highly similar to NUP98/HD fusions such as NUP98-HOXA9 or NUP98-PMX1. NUP98/HHEX expression in primary murine bone marrow cells led to aberrant self-renewal capacity and block of normal differentiation as shown in serial replating assays and liquid cultures. Trasplantation of bone marrow cells retrovirally expressing NUP98/HHEX led to acute leukaemia with extensive infiltration of leukaemic blasts expressing myeloid ($Gr1^+$, $Mac1^+$) and B-cell lineage markers ($B220^+$). Comparative gene expression profiling demonstrated NUP98/HHEX deregulated several genes that are targets of NUP98/HOXA9 not only in the mouse model but also in human NUP98/HHEX leukaemia. Conclusions Patient 1: LOC348801, a new NUP98 partner in the 46,XY,t(3;11)(q12;p15), encodes for a protein with 178 aminoacids. Its functional characteristics are still unknown. Patient 2: HHEX , a new NUP98 partner, is a member of homeobox family gene. *In vitro* and *in vivo* experiments showed the leukemogenic mechanism and target of NUP98/HHEX fusion in AML. Supported by: MIUR, FIRB and Fondazione Cassa di Risparmio di Perugia. *We wish to thank Dr M Rocchi (University of Bari, Italy) for providing DNA clones.*

P015

PERFORIN MUTATIONS IN ADULT PATIENTS WITH VIRUS-RELATED LYMPHOPROLIFERATIONS AND PERIPHERAL CYTOPENIA AND WITH MARROW FAILURE SYNDROMES, ASSOCIATED WITH HEMOPHAGOCYTOSIS.

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Perforin is a cytolytic protein present in granules of cytotoxic T lymphocytes and natural killer cells playing an important role in immunosurveillance. Homozygous or compound heterozygous mutations have been identified in patients with familial hemophagocytic lymphohistiocytosis (FHL), a fatal autosomic recessive disorder of early childhood. Protein mutations have also been identified in disorders sharing common features with FHL, like T-cell non Hodgking lymphomas (T-NHLs), Autoimmune Lymphoproliferative Syndrome (ALPS) and its variants; bone marrow failure with hemophagocytosis; lymphomatoid granulomatosis and a case of chronic active Epstein-Barr virus (EBV) infection. All the mutations have been described in the coding region (exons 2 and 3). We have studied 19 patients, including 2 with viral-induced hemophagocytosis; 2 with bone marrow failure and hemophagocytosis, one of whom complicated by EBV- associated pneumonia; 9 with T-NHL; 3 with unusually persistent EBV reactivations following solid organ transplantation; 2 with lymphomatoid granulomatosis; 1 with recurrent EBV reactivations, as detected by persistently high EBV DNA in plasma, associated with hemolytic anemia (HA). Genomic DNA was isolated from the peripheral blood mononuclear cells (PBMCs); the coding regions of the Perforin gene were amplified by polymerase chain reaction (PCR) and directly sequenced. All the patients show the Perforin in the germline structure, except for the patient with recurrent EBV reactivations, associated with HA. This patient shows the heterozygous mutation C272T in exon 2 causing substitution of an Alanine with a Valine in position 91, while the FAS gene was in wild type configuration. In our selected group of patients without FHL we have found a low frequency of Perforin mutations (5,2%). The literature describes a pathological role of A91V only when is combined with other mutations in the Perforin gene or Fas gene. Further studies are undergoing to investigate whether this mutation, alone, could have a role in the persistent EBV reactivations associated with hemolytic anemia in an immunocompetent individual.

P016

RAPID DETECTION OF CEBPA MUTATIONS IN ACUTE MYELOID LEUKEMIA PATIENTS BY DENATURING-HPLC

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Background. In normal karyotype AML alterations at molecular level represent prognostic factors which can drive therapeutic intervention. The presence of CCAAT/enhancer binding protein-alpha (CEBPA) gene mutations is considered a favourable prognostic factor but the alterations can occur in all the 1200 base pare long gene and can be of different type so making the molecular analysis highly demanding. *Aims.* To develop a rapid and cost effective method for mutation detection in CEBPA gene and compare it with the most commonly used sequencing method. *Methods.* The intronless CEBPA gene was PCR amplified in three overlapping fragments (A, B and C) from 109 DNA samples derived from NK-AML patients. Each fragment was analysed for sequence alteration on Denaturing–HPLC (DHPLC) and sequenced in parallel. *Results.* DHPLC analysis revealed 11 out of 109 (10%) sequence alteration in fragment A, 38% in fragment B and 7% in fragment C (DHPLC-positive). All DHPLC-positive and negative PCR products were sequenced giving a 100% concordance for DHPLC-positive (A, B and C fragments) and DHPLC-negative for B and C fragments. We obtained 99% concordance for DHPLC-negative fragment A due to a point mutation occurring in homozygous status in one patient. DHPLC technique cannot reveal sequence alterations in homozygous status but this condition rarely occurs for CEBPA gene at diagnosis. However, homozygous alterations can be detected by DHPLC by mixing patient samples with

CEBPA gene amplification from normal donor producing an artificial heterozygous product. Analysis of sequence alterations, confirmed the presence of out of frame insertions or deletions in fragment A and in frame insertions or deletions in fragment C. In fragment B we found only one mutation while other sequence changes represent a base substitution polymorphism and a recently described 6 nucleotide duplication polymorphism. Overall, we identified a CEBPA mutation in 15% of analysed patients with 5 of them presenting a double mutation in fragment A and C. Patients enrolled in intensive chemotherapy protocols in our institution bearing CEBPA mutations had a better overall survival than patients without this molecular alteration (p=0.01). Conclusions DHPLC is an easy, rapid and cost effective method for CEBPA analysis. This technique can represent a valid alternative to the most commonly used sequencing method to identify alterations in CEBPA gene which represent a favourable molecular marker in normal karyotype AML.

P017

P27 REGULATION IN PERIPHERAL T-CELL LYMPHOMA NOT OTHERWISE SPECIFIED

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Background. Peripheral T-cell lymphoma not otherwise specified (PTCL/NOS) is the most common T-cell lymphoma; however, it remains a complex entity showing great variety regarding morphology, immunophenotype and clinical behaviour. In particular, these tumours are not still clearly defined by genetic analyses: in fact, many alteration were found, but no single genes were demonstrated to have a pathogenetic role. Recently, gene expression profiling (GEP) allowed the identification of PTCL/NOS-associated molecular signatures, leading to better understanding of their histogenesis, pathogenesis and prognostication. In particular, proliferation control turned out to be strongly affected, being a high proliferation index associated to unfavourable prognosis. P27, encoded by CDKN1B, is a member of the cyclin-dependent-kinase inhibitors family (CDKIs). Particularly, p27 is involved in G1 cell cycle arrest, its alterations possibly resulting in loss of normal cell cycle control, and neoplastic transformation. *Methods.* We investigated the possible involvement of p27 in cell cycle deregulation in PTCL/NOS. We performed GEP of 28 PTCLs/NOS, and 20 samples of normal T-cell subpopulations, by using the Affymetrix HG-U133 2.0 plus microarray. Furthermore, we studied the expression of p27, cyclin-E (CCNE1) and Ki-67 by immunohistochemistry (IHC) on tissue micro-arrays (TMAs) containing 98 PTCL/NOS cases. Finally, we performed direct sequencing of CDKN1B in 81 PTCLs/NOS. Results. First, GEP showed comparable CDKN1B expression levels in PTCLs and controls, with direct and inverse correlation to CCNE1 and MKI67, respectively. Second, IHC confirmed, in most instances, physiological inverse relation between p27 and Ki67 expression (p<0.02). Conversely, in almost a third of cases, we appreciated an apparent non-physiological balance between the two molecules. However, the two proteins were indeed co-expressed by a limited number of cells in 5 cases only. In such instances, of note, we excluded an aberrant expression of CCNE1, which was reported to overcome p27 function in Ki67$^+$/p27$^+$ neoplastic cells. Finally, direct sequencing did not indicate somatic mutations, but rather known polymorphisms of CDKN1B, not related to its expression. *Conclusions.* We conclude that p27 seems to be regulated in PTCL/NOS as in normal T-lymphocytes. Whether its co-expression with Ki-67 in few cells in a strict minority of cases might represent a pathologic phenomenon deserves future investigations.

P018

MOLECULAR MONITORING IN CORE BINDING FACTOR AML PATIENTS UNDERGOING AN AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

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The core binding factor (CBF) acute myeloid leukemia (AML) is included in the good prognosis category. Most patients achieve complete remissions (CR) after induction and long-term remission rates of 60-70% have been reported after an autologous transplant. Despite these encouraging results, 20-30% of patients relapse. The aim of our study was the evaluation of minimal residual disease by real-time reverse transcription polymerase chain reaction (RQ-PCR) in 20 consecutive adult CBF-AML patients submitted to an autologous peripheral blood stem cell transplantation (PBSCT) between 1999 and 2007: 12/20 showed a CBFbeta/MYH11 fusion transcript and 8/20 an AML1/ETO transcript. RQ-PCR was performed on bone marrow cells at diagnosis and molecular monitoring was performed after the induction, pre-transplant, on the apheretic products and every three months after the graft. Concerning the CBFbeta/MYH11 patients, 9/12 are in continuous complete remission (CCR) with a median follow-up of 44 months (range: 3-102), while 3/12 have relapsed after 3, 8 and 11 months from PBSCT, respectively. All patients received negative aphereses. No significant differences in median levels of fusion transcript at diagnosis and after induction were found between relapsed and non relapsed patients. However, the 3 relapsed patients never reached a <1 log cut-off level at any time and a >1 log rise in transcript level was predictive of the hematological relapse shortly after. On the other hand, all patients in CCR reached a <1 log cut-off level, 2 after consolidation and 6 after PBSCT. Currently, 8 patients are in molecular remission. In the AML/ETO patients, 7/8 patients received negative aphereses. One patient obtained a molecular remission after consolidation, but he showed an extramedullary relapse 5 months after the graft. 7/8 patients obtained a molecular remission after PBSCT at a median time of 2 months (range: 1-31) and 6 of them are in CCR with a median follow-up of 82 months (range: 55-97), while the patient who received a positive apheresis showed a molecular relapse 3 months later followed by an hematological relapse after 7 months. The disease-free and the overall survival of the 20 patients are 72% and 76%, respectively. Our data confirm the good prognosis in CBF-AML patients undergoing an PBSCT and the value in CBFbeta/MYH11 of a cut-off level during the follow-up which allows us to identify patients with a high risk of relapse. More studies are necessary in AML/ETO patients.

P019

IN VITRO ACTIVITY OF FOLATE ANTAGONISTS AGAINST METHOTREXATE-RESISTANT CCRF-CEM HUMAN T-LEUKEMIA SUBLINES AND RELATIONSHIPS WITH FOLATE PATHWAY GENE EXPRESSION PROFILES

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The *in vitro* cytotoxic activity of several antifolates was evaluated in human T-leukemia cell lines CCRF-CEM with acquired resistance to methotrexate (MTX) by virtue of different well-defined molecular mechanisms and in parental MTX-sensitive cells. The antifolates included a polyglutamatable (MTX) and a lyphophilic (trimetrexate, TMTX) inhibitor of dihydrofolate reductase (DHFR), two polyglutamatable inhibitors (CB3717 and raltitrexed, RTX) a non-polyglutamatable classical inhibitor (ZD9331) and a lipophilic inhibitor (AG337) of thymidylate synthase (TS), a polyglutamatable inhibitor (LY309887) of glycinamide ribonucleotide formyltransferase (GARFT) and a multitargeted antifolate (pemetrexed, MTA) inhibitor of TS, DHFR and GARFT. Drug activity was evaluated with the standard MTT proliferation assay measuring absorbance at 540 nm using a microplate reader after exposing cells to antifolate drugs for 72 hr. As expected, CCRF-CEM/BO cells (resistant by virtue of impaired MTX transport) displayed a higher level of resistance to this drug as compared to CCRF-CEM/R1 cells (resistant by virtue of DHFR gene amplification) (resistance indexes 88.7 and 25.0, respectively), while CCRF-CEM/P30 cells (resistant due to impaired polyglutamylation) were still sensitive to long term (72 hr) exposure to MTX. In CCRF-CEM/R1 cells partial cross-resistance to TMTX and MTA was observed while an unsubstantial change of *in vitro* potency was observed for antifolate drugs with a site of action different from DHFR (CB3717, RTX, ZD9331, AG337 and LY309887). CCRF-CEM/BO cells displayed partial cross-resistance to MTA and RTX, but were sensitive to lipophilic antifolates (TMTX and AG337) or antifolates mainly transported by RF-alpha (CB3717, LY309887). CCRF-CEM/P30 cells were slightly resistant to highly polyglutamatable antifolates RTX and CB3717 following a 72 hr exposure. No differences in the cell growth inhibitory effects of TMTX, AG337, ZD9331 and LY309887 as compared to parental CCRF-

CEM cells were observed following long term exposure. The data demonstrate that newer antifolates may have greater *in vitro* activity than MTX against human T-leukemia cells and may have potential for clinical circumvention of MTX resistance. The role of molecular determinants in antifolate cytotoxicity was also investigated in CCRF-CEM MTX-resistant and sensitive cells. Gene expression studies by quantitative real time RT-PCR demonstrated correlations between antifolate sensitivity/resistance and expression of genes involved in their transport, metabolism and site of action, suggesting that sensitivity/resistance to these drugs may be predicted on the basis of pharmacogenomic profiles. Supported by a grant from Associazione Giacomo Onlus.

P020

PERSISTENT AKT INACTIVATION AND C-ABL NUCLEAR IMPORT FOLLOWING MTOR INHIBITION COMPLEMENT IMATINIB MESYLATE EFFECTS ON CHRONIC MYELOID LEUKEMIA

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Mammalian TOR (mTOR) belongs to the family of phosphatidylinositol 3-(PI) kinase-related kinases (PIKKs). Its constitituve activation by p210 BCR-ABL oncogenic tyrosine kinase (TK) up-regulates the translation of growth and survival factors through the activation of eukaryotic initiation factor 4E (eIF4E) and ribosomal protein S6, and is thereby involved in the pathogenesis of Chronic Myeloid Leukemia (CML) (Prabhu et al, Oncogene 2007; 26:1188-1200). Moreover, it may compensate Imatinib mesylate (IM) inhibitory effects and contribute to the disease progression towards a drug-resistant phenotype (Burchert et al, Leukemia 2005; 19:1774-82). Here we demonstrated that the mTOR inhibitor RAD001 (Everolimus from Novartis) enhances IM cytotoxic effects on murine myeloid progenitors (32D) stably transduced with a temperature-sensitive p210 BCR-ABL construct (whose protein owns tyrosine kinase activity only at the permissive temperature of 33°C)(Brusa et al, Br J Haematol 2005; 132: 359-69). Multiple mechanisms are involved in the two drugs synergism, including the de-phosphorylation of mTOR, the reduction of mTORC2 complex and of its components: Rictor and SIN1/MIP1. Notably, SIN1/MIP1is a component of AKT pathway that positively controls AKT activating phosphorylation at Ser473 near the C-terminal (Jacinto et al, Cell 2006;127:125-37). Accordingly, RAD001 precludes AKT re-phosphorylation at Ser473 occurring after longer intervals (24 hrs) of exposure to IM and, in addition, promotes a significant reduction of AKT phosphorylation at Thr308 thereby attaining its complete inactivation. RAD001 elicits the retrograde AKT inhibition in 32D parental cell line and 32D BCR-ABL-expressing cells kept at the non-permissive temperature (39°C) for p210 TK activity. Furthermore, IM and RAD001 association enhances the nuclear translocation of normal c-ABL protein (not rearranged with the BCR product) through mechanisms proceeding from its release from the cytoplasmatic binding to 14-3-3 sigma scaffolding protein. AKT persistent inactivation and c-ABL nuclear import and activation implement growth arrest and apoptotic death of leukemic progenitors. In conclusion, our results support that the association of mTOR inhibitor RAD001 to IM affects signals involved in the proliferation and survival advantage of leukemic progenitors as well as in the development of a drug-resistant phenotype. It may therefore considered for the treatment of CML patients.

P021

SLUG TRANSCRIPTION FACTOR IN CHRONIC MYELOID LEUKEMIA PROGRESSION TOWARDS A DRUG RESISTANT PHENOTYPE

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SLUG, a SNAIL-related zinc-finger transcription factor, is a critical component of mesenchymal cell differentiation and response to antineoplastic drugs. It is transcriptionally induced by p53 in response to DNA damage and protects hematopoietic progenitor cells from apoptotic death by repressing the BH3-only pro-apoptotic protein PUMA (Wu et al Cell 2005; 123: 641-53). Here we investigated the impact of SLUG on the development of Imatinib mesylate resistance in Chronic Myeloid Leukemia (CML). In preliminary experiments we found SLUG overexpression associated with the expression of activated p210 BCR-ABL tyrosine kinase (TK) in individual cell clones generated from murine myeloid progenitor cell line 32D stably transduced with a BCR-ABL construct. The findings support SLUG involvement in BCR-ABL leukemogenesis (Pérez-Mancera *et al.*, Oncogene 2005; 24: 3073-92). A further increase of SLUG expression was then seen in BCR-ABL-expressing 32D cell that developed an IM-resistant phenotype either spontaneously or in consequence of growth factor (IL-3) ligand to cognate receptor (Mancini *et al*., Leuk Res 2007; 31:979-87). SLUG over-expression associated with IM resistance was confirmed in Ba/F3 cell lines expressing the BCR-ABL either in its wild type (wt) conformation or harbouring point mutations coding for E255K and T315I p210 isoforms. As expected, it was the cause of a very significant reduction of PUMA transcript and protein. The critical role of SLUG in BCR-ABL-induced transformation and drug resistance came from experiments with a specific silencing of SLUG through the following siRNA duplex oligoribonucleotides: 1) AUCAGAAUGGGUCUGCAGAUGAGCC, 2) GGCUCAUCUGCAGACCCAUUCUGAU. Fourty eight hrs exposure to this siRNA promoted, in fact, PUMA induction followed by massive apoptosis induction in BCR-ABL-transduced 32D and Ba/F3 cells either IM-sensitive and IM-resistant. SLUG over-expression in CML cells may arise from the constitutive activation of mitogen-activated protein kinase (MAPK) pathway. In particular, the enhancement of p42/44 MAPK activity in response to IM may contribute to the development of drug resistance (Chu et al, Blood 2004; 103: 3167-74). Accordingly, complete elimination of leukemic cells may be attained by SLUG repression by MAPK inhibitor and IM association. Notably, the major effects of MAPK inhibitor and IM association are expected at the level of leukemic stem cells, whose survival is controlled by SLUG downstream of Stem Cell factor/c-Kit signalling pathway (Pérez-Losada *et al.*, Blood 2002; 100: 1274-86).

P022

DIFFERENTIAL CELLULAR LOCALIZATION OF TFR2 ISOFORMS

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The transferrin receptor 2 (TFR2) is a transmembrane protein involved in iron metabolism since TFR2 gene mutations are responsible of type 3 Hereditary Hemochromatosis (HH). Tfr2 protein is a member of the transferrin receptor family, showing moderate homology to transferrin receptor (TFRC) and it is able to bind transferrin even if with lower affinity than TFRC. Two alternative transcripts of TFR2 have been reported, alfa and beta. TFR2 alfa mRNA is highly expressed in the hepatocytes while the beta transcript has a low ubiquitous expression (Kawabata *et al*., 1999). The beta form is identical to the alfa one but it lacks the transmembrane and cytoplasmic domains; it was considered an alternative splicing form and no further studied. Nevertheless, clinical observations support the hypothesis that the beta form as an important functional role since patients with mutations not compromising its production have a milder phenotype compared to alfa and beta non producing HFE3 patients. In order to investigate *in vitro* the expression pattern and the cytoplasmic localization of the two alternative forms they have been cloned in an expression vector and transfected in HEK293T and COS7 cell lines. Alfa and beta expressing cells cultures have been treated with holo and apotrasferrin, iron citrate and dexferroxamine for 24 hours. Immunofluoresce experiments demonstrated a different alfa and beta forms cellular localization in untreated cells 24 hours after transfection being the former localized in plasma membrane, as expected, while TFR2 beta form was mainly intracytoplasmic. Twenty-four hours after treatment with olotransferrin TFR2 alfa remains on the plasma membrane while the beta form is localized under the cellular membrane forming well defined spots. Preliminary results on the other Hemochromatosis genes transcription pattern (HEPC, FPN1 HFE and HJV) revealed a different transcription modulation of the same gene in alfa *vs.* beta

transfected cells as a response to the same treatment. On the whole these results suggest a functional role for the TFR2 beta form in iron sensing, since the isoform responds to iron increasing moving from cytoplasm to a well defined portion of the plasma membrane. This is consistent with previous data suggesting a preferential localization of TFR2 protein in specialized cellular membrane domains (Calzolari *et al.*, 2006)

P023

CHRONIC MYELOMONOCYTIC LEUKEMIA: IDENTIFICATION OF MOLECULAR ABNORMALITIES AND MONITORING OF RAS MUTATIONS-DRIVEN DISEASE PROGRESSION BY ALLELE-SPECIFIC PCR

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CMML is a rare, heterogeneous malignancy whose hallmark is absolute monocytosis in blood. The cut off of 13×10^9 white blood cells (WBC)/L has been established to distinguish between a dysplastic (MD) and a proliferative (MP-) variant of the disease: yet, whether they represent phases of the same disease or independent disorders remains unclear. Lessons learned from animal models along with analysis of pts samples point to the role of RAS mutations, hypermethylation of p15INK4b promoter and, to a lesser extent, JAK2V617F substitution, FLT3-ITD, PDGFRbeta rearrangements and Bid deletion in CMML development. Nonetheless, to date no effective therapy is available and prognosis of most pts remains poor. We aimed to identify molecular differences between MD- and MP-CMML and set up allele-specific PCR (ASP) for early identification of RAS genes mutations. Genomic DNA was extracted from mononuclear cells of 48 CMML pts (21 with MD-CMML, 25 with MP-CMML and 2 with border-line disease). A point mutation of N- or K-RAS was detected by direct sequencing in 0/21 MD-CMML and in 6/25 MP-CMML pts: 3 harboured the N-RASG12D, 1 the N-RASG12R, 1 the novel N-RASG60E, and 1 the K-RASG12R substitution. Noteworthy, the G60E mutation was detected after progression from MD- to MP-CMML and we demonstrated higher *in vitro* activity of the mutant protein compared to wild-type (wt) N-Ras, while the N-RASG12R substitution was identified in a pt with a prior history of CML, when no such mutation could be recognized. Also, ASP detected the G12D allele in two pts with border-line disease and wt RAS by sequencing. Follow-up of one of these pts, who experienced a marginal increase of WBC to more than 13×10^9/L, unveiled a rise of G12D allele levels at an earlier time than it was detectable by sequencing. In agreement, in the pt with the G60E substitution, ASP was able to detect low levels of mutant allele also in a sample withdrawn earlier, when the pt was in MD- phase. Set up of ASP for N- and K-RASG12R mutations is presently ongoing. Finally, the JAK2V617F substitution and FLT3-ITD were found in 4 and 1 MP-CMML pts, respectively, while screening for p15 promoter methylation is in progress. Of note, none of these molecular abnormalities (which were mutually exclusive in pts with MP-CMML) were detected in our MD-CMML group. Altogether our results imply a major role for RAS mutations in progression from MD- to MP-CMML, and strongly indicate ASP as a powerful tool for disease monitoring.

Cytogenetics

P024

MINIMAL RESIDUAL DISEASE MONITORING IN CML PATIENTS: COMPARISON BETWEEN AUTOMATED FISH ANALYSIS AND RQ-PCR

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Background. Real-time quantitative PCR (RQ-PCR) is acknowledged as the gold standard approach for MRD monitoring in CML patients during follow up. *Aims.* To compare RQ-PCR method with a novel FISH analysis approach based on a fully automated FISH slide scanner and image analyzer (Duet BioView, Israel) for picking up rare cell events. *Methods.* Fifty-one CML patients in clinical and cytogenetic remission following imatinib (IM) therapy (44 patients), or bone marrow transplantation (BMT; 7 patients) were investigated. Ninety samples, 75 from bone marrow and 15 from peripheral blood, were tested with both RQ-PCR using TaqMan protocol (Applied BioSystems, USA), and Duet BioView FISH slide scanner by scoring 1600-4500 cells for BCR-ABL rearrangement using a dual-color, dual-fusion FISH probe combination (Kreatech, Denmark). Leukemic cell levels in the samples were arbitrarily grouped in three classes: >1%; 0,99%-0,04%; and <0,04% of scored cells. *Results.* FISH/RQ-PCR concordance was 100% for class >1%, 96% for class 0,99%-0,04%, and 84% for class <0,04% leukemic cells. Samples with FISH/RQ-PCR discordant results showed leukemic cells as evidenced by FISH close to the detection limit of FISH procedure itself (i.e. 0,04%). The discordant cases had <0,01% BCR-ABL transcript level and no recurrence of 'molecular' disease in the following 18 months of treatment. In two patients FISH analysis unravelled 3/1900 (0,16%), and 8/3800 (0,21%) leukemic cells carrying two copies of BCR-ABL fusion, i.e. double Ph, which are undistinguishable from those with a single copy of BCR-ABL rearrangement when investigated by RQ-PCR. IM dose escalation (800 mg/day) resulted in disappearance of double BCR-ABL leukemic cells, which are still absent 24 and 30 months from high-dose therapy start, respectively. Peripheral blood samples also showed FISH/RQ-PCR concordant results when >3000 cells were scored by FISH. *Conclusions.* Present data show that automated FISH analysis by scoring >1600 bone marrow or >3000 peripheral blood cells/sample provides useful information for MRD monitoring in CML patients, being FISH results largely overlapping with those obtained with RQ-PCR approach. Furthermore, early occurrence of double Ph-positive cells can also be recognized allowing appropriate therapy protocol modification.

P025

INFLUENCE OF ADDITIONAL CHROMOSOME ABNORMALITIES TO PH ON RESPONSE TO IMATINIB MESYLATE THERAPY IN EARLY CHRONIC PHASE IN CHRONIC MYELOID LEUKEMIA. (A GIMEMA WP ON CML ANALYSIS)

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Introduction. Chronic Myeloid Leukemia (CML) is characterized by the reciprocal translocation t(9;22)(q34;q11). At diagnosis, the Ph chromosome occurred as the sole abnormality in the majority of cases; additional chromosome abnormalities (ACA) can appear in about 7-10% of cases, according to several series. The clinical impact of these changes can depend on the treatment. We evaluated the response to Imatinib Mesylate (IM) in CML patients (pts) with ACA. *Methods.* Five hundred and sixty pts in early chronic phase (CP) were enrolled into three multicentric national clinical trials of GIMEMA WP on CML. These pts were monitored by conventional cytogenetics (CC) and molecular analysis (3-6-12 months) and then every 6 months, during IM treatment. Median observation time was 30 months. *Results.* At enrollment, 480 pts were evaluable by CC and 21 pts (4.4%) showed ACA: loss of chromosome Y in 9 pts (42.9%), trisomy of chromosome 8 in 3 pts (14.3%), other single abnormalities in 7 pts (33.3%) and 2 ACA in 2 pts (9.5%). The 2 groups of pts, with or without ACA, were similar for age, Sokal risk and IM dose. At 12 months, 12/21 pts (57% *vs.* 84.5% of pts without ACA; p=0.0034) achieved complete cytogenetic response (CCgR), 4 (19%) partial response (PCgR) and 5 (24%) failed the treatment. Seven pts (33.3%) reached Major Molecular Response (MMolR). Of 3 pts with trisomy 8, only 1 reached CCgR, 1 PCgR and the last one was in mCgR. Of 8 pts with loss of Y chromosome, 5 (62.5%) reached CCgR, 2 PCgR and 1 didn't show any response (NR). Six out of 7 pts (85.7%) with single and different abnormalities reached CCgR, but 1 patient lost the cytogenetic response after 24 months. The 2 pts with 2 ACA discontinued IM therapy because of unsatisfactory therapeutic effect: 1 in NR at 6 months and the other one in mCgR at 12 months. As regards best response, 15 pts (71.4% *vs.* 92.6% of pts without ACA; p=0.0049) achieved CCgR. Of these, 12 pts reached MMolR, too. Seven pts failed the treatment (33.3% *vs.* 10.9% of pts without ACA; p=0.0071). *Conclusions.* The results of this large series of CML pts in early CP confirm that the occurence of ACA plays a pivotal role on the prognosis in terms of CgR rate. Moreover, complex karyotype (presence of two additional abnormalities) seems to retain an adverse outcome during IM therapy. *University of Bologna (RFO), Fondazione del Monte di Bologna e Ravenna, European LeukemiaNet founds, MIUR PRIN 2005, Bologna AIL.*

P026

PROGNOSTIC SIGNIFICANCE OF MOLECULAR MARKERS IN NORMAL KARYOTYPE -AML: A SINGLE CENTER EXPERIENCE

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Background. Cytogenetic alterations well define the outcome of Acute Myeloblastic Leukemia (AML). In Normal Karyotype (NK) AML other molecular markers have been proposed, but the clinical significance of some of them is still not well defined. *Aims and methods*. To verify the clinical significance of molecular markers in predicting the outcome of NK-AML, diagnostic samples derived from 105 patients (age<60y) enrolled in 4 sequential intensive chemotherapy protocols were analyzed for FLT3-ITD, FLT3-TKD, NPM1, WT1 (exon 7 and exon 9), MLL-PTD and CEBPA mutations. *Results*. The prevalence of each mutation was the following: FLT3-ITD 32% (30/94), FLT3-TKD 7% (7/97), NPM1 49% (44/90), WT1 9% (7/77), MLL-PTD 6% (3/53), CEBPA 16% (8/51). The CR rate of this group of patients was 88%. With a median observation time of 21,1 months the Overall Survival (OS) and Disease Free Survival (DFS) of this group of NK-AML patients were 40% and 43%, respectively. By univariate analysis, OS was decreased in the presence of FLT3-ITD (26% *vs.* 48%, p=0.015) while it was apparently increased by the presence of CEBPA mutation (100% *vs*. 44%). Patients carrying WT1 gene mutations showed an inferior OS (28% compared to 43% of WT1 negative patients) but this difference did not reach a statistical significance (p=0.4). The DFS was significantly inferior in FLT3-ITD positive patients (30% *vs.* 51%, p=0.006) and a 75% DFS was seen in CEBPA positive patients compared to 48% in CEBPA negative patients (p=0.2). The probability to achieve a CR was superior in NPM1 positive patients (93% *vs.* 78%, p=0.04) while no significant difference was associated with the presence of other mutations. By multivariate analysis (CEBPA and MLL-PTD were excluded for the limited number of patients), the presence of FLT3-ITD independently predicts a worse DFS (HR 2.19, CI 1.03-4.66, p=0.041). On the contrary, the presence of NPM1 mutation predicts a better CR rate (OR 6.17, CI 1.01-37.52, p=0.048) and showed also a positive trend for a better DFS (HR 0.48, CI 0.22-1.06, p=0.07) and a better OS (HR 0.54, CI 0.26-1.1, p=0.09). The presence of WT1 gene mutations was associated with a worse OS (HR 2.8, CI 0.86-9.5, p=0.08). *Conclusions*. In a cohort of NK-AML patients, we confirmed the adverse prognostic impact of FLT3-ITD and WT1 mutations, and the favorable outcome associated with CEBPA and NPM1 mutations. All these markers should be identify in each case in order to better define the prognostic profile of each patient.

P027

IN MYELODYSPLASTIC SYNDROMES AND ACUTE MYELOID LEUKEMIA MONOSOMY 20 MASKS COMPLEX CHROMOSOMEC 20 DEFECTS. A MOLECULAR CYTOGENETIC STUDY ON 10 PATIENTS

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Deletions of the long arm of chromosome 20 (20q-) are recurrent karyotype defects in patients with MDS and AML. Conventional cytogenetic, fluorescence in situ hybridisation (FISH) and molecular studies have identified a common deleted region (CDR) of about 2.6 Mb on the long arm of this chromosome. Moreover, the L3MBTL tumor suppressor gene (TSG), which resides within this CDR, has been identified as the gene potentially responsible of the pathogenesis of 20q- MDS/AML even if its role has not been precisely defined yet. However, very recent FISH studies have revealed that 20q deletions or unbalanced translocations are almost always accompanied by the retention of the most proximal long arm segment of chromosome 20. Thus, it has been suggested that this chromosome 20 region might contain either a gene or a sequence crucial for MDS/AML development. The aim of the present study was to verify this hypothesis in 10 MDS/AML patients carrying a complex karyotype, defined by the presence of ≥3 chromosome defects including either a 20q deletion or a monosomy 20. According to FAB classification 2 patients were classified as refractory anemia (RA), 5 as refractory anemia with excess of blasts (RAEB), 2 as RAEB in transformation (RAEB-t) and one as AML. According to WHO classification 2 patients were classified as refractory cytopenia with multilineage dysplasia (RCMD), 2 as RAEB-1, 3 as RAEB-2 and 3 as AML. CC and FISH studies were performed as already reported. FISH analyses were carried out on mitotic figures with the D20S108 (mapped in 20q12) and with the WCP20SO probes from Vysis (Abbott Molecular/Vysis, North Chicago, IL, USA) and with an arm chromosome painting (ACP) 20q probe from QBiogene (Qbiogene Inc., Carslbad, CA, USA). The D20S108 probe provided only one signal in 7 patients and ≥4 signals in 3 patients. So, a 20q deletion was revealed in 7 patients and an amplification in 3. When the WCP20 probe was applied, 8 patients showed a normal chromosome 20 and various fragments of number 20 on other chromosomes, one patient a pentasomy 20 and one patient a true mono-

somy 20. When the ACP 20q probe was applied, 7 of the former 8 patients showed a normal long arm of chromosome 20 along with fragments of 20q. Therefore, this probe confirmed that all fragments, which the WCP20 probe had already identified as material derived from number 20, truly belonged to 20q. Moreover, in 3 of the 7 patients with 20q amplification the ACP probe revealed that the DS20108 probe was contained within the amplified region. Finally, the ACP probe confirmed pentasomy 20q in one patient and true monosomy 20 in the last one. In conclusion, our data confirm that i) true monosomy 20 is a very uncommon event in MDS/AML, ii) in most patients an apparent monosomy does occur since material from chromosome 20q is always retained, iii) the common feature in all our patients is 20q amplification (observed in 7 patients) rather than band 20q12 deletion (observed in 4 patients), a datum which strengthens the suggestion that a still undetected oncogene might be mapped within the amplified area. So, our future goal will be to define the extent of the amplified area searching for a possible oncogene.

P028

FISH ANALYSIS IN EXTRAMEDULLARY PLASMOCYTOMAS

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Extramedullary plasmocytomas account for 3% of plasmacells tumor and there is an impression that the incidence is increasing. It may be primary solitary plasmocytomas or may occur during multiple myeloma (MM) progression, either as an isolated phenomenon or concurrent with systemic relapse. Informations on incidence, treatment and genetic profile are scarce. We report genetic FISH-analysis of two extramedullary MM relapses and one primary solitary plasmocytomas. Any cases didn't show bone marrow involvement. Plasmacells (PCs) collected from extramedullary mass lack CD56 antigen. FISH analysis with D13S319, LSI IGH/CCND1, LSI IGH/EGFR3, c-myc, CEP 8 and p-53 probes (Vysis, Downers Grove, IL) was performed on PCs to detect respectively deletion of chromosome (#) 13, translocation t(11;14), t(4;14), # 8 polysomy and/or c-myc amplification and p-53 deletion. All cases showed # 13q deletion and absence of p-53 deletion. Both extramedullary MM relapses, in addition to del(13q), showed c-myc amplification in 25-30% of PCs; one case showed also # 8 polysomy (12-15% of PCs), and t(11;14) (79% of PCs). Primary solitary plasmocytomas, in addition to del(13q), showed loss of # 8 (74% of PCs) and monoallelic deletion of 14IGH locus (80% of PCs). C-myc/IGH translocations and c-myc rearrangements are reported in MM (~10%) more than c-myc amplification. In literature recent evidence of trisomy 8 as one of commonest abnormalities in myeloid sarcoma is reported. Our findings on c- myc amplification and/or # 8 polisomy in extramedullary plasmocitomas suggest that this genetic events may play an important role in the development of extramedullary and more aggressive diseases. Further studies are thus necessary in order to better define the genetic profile of the extramedullary diseases.

P029

A COMPLEX T(10;11) TRANSLOCATION INVOLVING THE MLL GENE IN A PATIENT AFFECTED WITH AML M5

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The t(10,11)(p12;q23) translocation is a recurrent and rare chromosomal abnormality in AML especially associated with a M5 FAB cytotype. Cytogenetic studies have demonstrated an heterogeneity in 10p breakpoints even if molecular analysis have revealed that the AF10 gene is constantly targeted by this translocation. Up to now, two types of chimeric transcripts the MLL-AF10 in the t(10;11)(p12;q23) and CALM-AF10 in the t(10;11)(p13;q14) have been isolated. In 1998 Taki et al identified the Abl-interactor 1 (ABI1) gene as a new MLL partner in the t(10;11) translocation. It was also stated that this chromosomal rearrangement is significantly associated with complex translocations including invins(10;11) and inv(11)t(10;11). Herein we report an additional female patient with a complex t(10;11) rearrangement and a M5 AML. She was forty-seven years old. Her blood count was the following: haemoglobin 9,4g/dl, WBC $34,2\times10^9$/L (blast cells 88%), Plts 7.0×10^9/L. Induction chemotherapy determined a complete remission (CR) which lasted six months. After the achievement of a second CR the patient was submitted to an allogeneic bone marrow transplant. Now she is alive and well ten months post-transplant. On clinical diagnosis cytogenetic analysis, carried out on bone marrow cells, revealed the following karyotype: 46,XX/46,XX,t(10;11)(p14;q23),-10, +der(10)t(10;11). FISH studies were performed with the following probes: LSI MLL and LSI ATM from Vysis (Abbott Molecular/Vysis, North Chicago, IL, USA), Whole Chromosome Painting (WCP) 11 and Arm Chromosome Painting (ACP) 10p from Q-Biogene (Qbiogene Inc., Carslbad, CA, USA). Moreover, in order to better define the rearranged chromosomal region the RP11-47P2 (mapping at 10p15.1), the RP11-79F9 (corresponding to 10p14), the RP11-13C4 and the RP11-36M5 (covering the PLZF3 gene at 11q23.2) BAC probes (kindly provided by the Wellcome Trust Sanger Institute, Cambridge, UK) were applied. The WCP 11 and the ACP 10p probes confirmed the t(10;11) translocation, the loss of the normal chromosome 10 and the duplication of der(10)t(10;11). When analysing chromosome 11 the ATM and the PLZF3 probes were in normal position revealing that neither gene was involved in the rearrangement. Instead, both the signals (red and green) forming the MLL break apart probe were translocated to chromosome 10p and split by an additional rearrangement. So, we hypothesized that the t(10;11) translocation occurred first and was followed by an additional defect. This possibility was further confirmed by the presence of chromosome 10 material inserted between the two MLL signals, an event which was interpreted as either an inversion or an insertion. When analysing chromosome 10 the RP11-79F9 green probe remained in the correct position, whereas the RP11-47P2 red probe was translocated to chromosome 11. Thus, in the initial t(10;11) the breakpoints on chromosome 10 fell within the region delimited by these two BAC probes. This region, which is about 1Mb long, does not contain any MLL partner gene previously described. Based on this datum and on the fact that either the AF10 or the ABI1 gene, located at 10p12, are MLL partners in the usually complex t(10;11) translocations we are currently trying to define the breakpoints associated with the secondary rearrangement on chromosome 10. In fact, these breakpoints because of their more centromeric position might truly involve either the AF10 or the ABI1 gene.

P030

CHROMOSOME ABNORMALITIES ADDITIONAL TO THE PHILADELPHIA CHROMOSOME AT THE DIAGNOSIS OF CHRONIC MYELOID LEUKEMIA. PATHOGENETIC AND PROGNOSTIC IMPLICATIONS

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Background. The secondary origin of the Ph chromosome in some patients has been demonstrated by the persistence of chromosome abnormalities after karyotypic conversion during Imatinib treatment (1, 2). We tried to verify the real incidence and the possible prognostic value of this finding in a larger population of patients. *Patients and methods.* Out of 54 Italian Cytogenetics Laboratories, 9 provided 15 patients. Nine were males and 6 were females. Median age was 55.5 years (extremes 35-80). One patient died 11 months after diagnosis, 1 is in blastic crisis and 1 in accelerated phase. *Results.* In 5 patients, the additional abnormalities persisted after the disappearance of the Ph-positive clone. Four patients had a High Sokal Score and 1 an Intermediate one. One patient is dead, all the other patients are alive and in complete cytogenetic remis-

sion. In 7 other patients with Low (4 patients) or Intermediate (3 patients) Sokal Score, both the Ph and the additional abnormalities disappeared, completely in 5 and partially in 2. Two patients of this group had a molecular relapse (on month +10 and on month +46, respectively). In the last 3 patients, all with a high Sokal Score, no cytogenetic response was observed. *Discussion*. The persistence of cytogenetic abnormalities in Ph-negative metaphases during Imatinib treatment, clearly suggests that they occurred as primary events (1, 2). Conversely, in most patients the additional chromosome abnormalities were present together with the Ph and either disappeared together with the Ph, in case of r Moreover, based on the data reported here, it seems that additional chromosome abnormalities may have a few prognostic value: they are frequently associated with a High or Intermediate Sokal Score; patients who show persistence of the chromosome abnormality alone during Imatinib therapy, may have a response even if with a High Sokal Score. Conversely, those in which the chromosome abnormality is always detected in association with the Ph chromosome respond to Imatinib if in the Low or Intermediate, but do not if in the High Sokal Score group. A larger series of patients is necessary to confirm these data.

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P031

WHAT BANDS ON THE LONG ARM OF CHROMOSOME 6, IN ADDITION OF THE 6Q21, ARE INTERESTED BY CYTOGENETIC ALTERATIONS IN B-CLL?

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Deletions affecting the long arm of chromosome 6 (6q) are among the most commonly observed chromosomal aberrations in lymphoid malignancies and have been identified as adverse prognostic factor in subset of solid tumors. The results reported in previous cytogenetic studies suggest that 6q is the site of tumor suppressor genes involved in the pathogenesis of malignant lymphoid diseases.

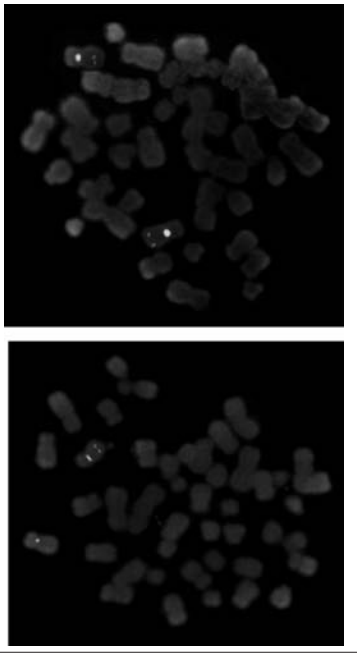

Figure 1.

The deletions of the long arm of chromosome 6 is know to occur at relatively low frequency (3-6%) in B cell chronic lymphocytic leukaemia (B-CLL) and the deletions are more frequently observed in 6q21. These cases of B-CLL carrying deletion of 6q21 shows clinicobiological features and outcome that should be allocated in an intermediate-risk category. Few data have been reported regarding other band on 6q interested by cytogenetic alterations in B-CLL. In the current study, begun to January, we have used four BAC clones mapping regions in bands 6q16, 6q23, 6q25, 6q27 as probes for fluorescence in situ hybridization (FISH) in twelve B-CLL cases to analyze the incidence and localization of 6q aberrations. Moreover a comprehensive set of commercially available probes was used, as follows: del(11q22-q23) ATM; +12, centromeric probe for chromosome 12; del(13q14), D13S319, D13S325; del(17p13), p53; 6q21. The FISH study was performed in nuclei and metaphases obtained after stimulation with a combination of CpG-oligonucleotide DSP30 and interleukin 2. Among twelve patients studied with B-CLL we identified two cases (16,6%) with 6q deletion. One deletion was found with the probe mapping in 6q23 and the same case showed deletion in 6q25 (case showing trisomy 12 and deletion of 17p13 detected by FISH); another one deletion was found with the probe mapping in 6q16 (case showing trisomy 12 detected by FISH). About these cases was available data of chromosome banding analysis. Cytogenetic analysis showed a complex karyotype with presence of two distinct clones in the case with deletion in 6q23 and 6q25, while in the case showing deletion in 6q16, chromosome banding analysis confirmed the results of FISH analysis in detecting trisomy 12. In the present study ninety patients will be analyzed to find what bands in addition of 6q21 are interested from cytogenetic alterations and to correlate the alterations observed to the clinical course.

P032

PROGRESSION OF A MYELODYSPLATIC SYNDROME, WITH NONRANDOM ANEUPLOIDY OF CHROMOSOME 21, TO ACUTE MYELOID LEUKEMIA WITH T(9;15)(Q22;Q22) AS THE SOLE CYTOGENETIC ABNORMALITY

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Myelodysplastic syndromes (MDS) are a group of eterogeneous stem cell disorders characterized by peripheral blood cytopenia and bone marrow dysplasia. Approximately 30% of cases of MDS progress to acute myelogenous leukaemia (AML). Abnormal number of chromosome, the aneuploidy, is the most common abnormality in cancer and expecially in MDS. The aneuploidy is proposed as the primary cause of the genomic instability of neoplastic and preneoplastic cells that destabilizes the karyotype and generates new chromosome rearrangements. Moreover, additional copies of chromosome 21 are frequently found in leukemic cells too and the high incidence of acute leukaemia in subjets with constitutional trisomy 21, suggests that chromosome 21 plays a particular role in leukemogenesis. In this report, we describe a case of 55 yo patient who received diagnosis of myelodisplastic syndrome. At diagnosis the bone marrow showed the presence of 15% of blasts and the cytogenetic analysis revealed an abnormal karyotype: 47,XY,+21[3]/41, XY[2]/46,XY[3]. THE PATIENT RECEIVED DIAGNOSIS OF Myelodysplastic syndrome IPSS 3. The patient received demethylating agent 5-azacytidine therapy for 6 months and he went into in complete hematologic remission, while the cytogenetic analysis performed on bone marrow cells revealed the following karyotype: 46,XY,t(9;15)(q22;q22), as the sole cytogenetic abnormality. After 5 months, the patient progressed to AML and the new cytogenetic analysis showed the t(9;15))(q22;q22) as the sole cytogenetic abnormality again. The Fluorescent in Situ Hybridization (FISH) analysis, utilizing the whole chromosome painting probe for chromosome 21 (Vysis) and the LSI 9q34 SpectrumAqua Probe (Vysis), confermed the trisomy of chromosome 21 on cytogenetic material of diagnosis and the t(9;15)(q22;q22) after remission and at progression to AML. The progression of MDS to AML is characterized by the karyotype changes. In our case the presence of trisomy 21 in MDS phase confirms the oncogenic mechanism of chromosomal aneuploidies and the implication of chromosome 21 in the process of leukemogenesis.

P033

ATYPICAL CHRONIC MYELOID LEUKAEMIA WITH T(9;22)(P24;Q11)

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Chronic Myeloid Leukemia (CML) is characterized in 85% to 90% of cases by the presence of the t(9;22)(q34;q11) that determine the BCR/ABL fusion gene. A further 5% to 10% of cases have variant translocations that involve one or more chromosomal regions in addition to bands 9q34 and 22q11, but there are also simple variants that tipically involve 22q11 or, less commonly, 9q34 region and a third chromosomal region. In majority of case the patients are found to be positive for BCR/ABL by molecular analysis. A minority of patients with clinical and haematological features suggestive of CML are Ph negative and BCR/ABL negative. These patients are classified as atypical CML that some time may have distinct gene fusions. The most frequent translocation observed in these patients are: t(9;12)(q34;p13) with ETV6/ABL fusion gene and the t(8;22)(p11;q11) with BCR/FGFR1 fusion gene. We describe the case of a patient with a CML-like syndrome, characterized by the t(9;22)(p24;q11) as the sole cytogenetic alteration. A 35-year-old male presented with fatigue and fever. The full blood examination revealed haemoglobin 10,1g/dl; the white blood cell count was $69{,}0\times10^9$/L; and platelets were 110×10^9/L. The bone marrow analysis revealed myeloid hyperplasia, while the cytogenetic analysis, performed on bone marrow cells, showed the t(9;22)(p24;q11) in 20 of 20 metaphases. The RT-PCR didn't show BCR/ABL rearrangement. To determine whether in this t(9;22) was involved the BCR gene, we performed Fluorescence In Situ Hybridization (FISH) using the probe LSI BCR/ABL dual-color, dual fusion (Vysis). The FISH studies analysis didn't show BCR/ABL fusion in 10 of 10 metaphase analized and in 100 interphase cells analyzed. Interestingly, in FISH analysis, the metaphases showed red signals (ABL) on both copies of chromosome 9; one large green signal (BCR) on the normal chromosome 22, with smaller green signals on the der (22) and on the short arm of der (9). These data suggested that the translocation break-point was in the BCR gene, that the 3' portion of BCR has been translocated to 9p24. It is known that JAK2 gene now implicated in myeloprolipherative syndromes is located on 9p24, we hypothesize that the may be created a new fusion gene (BCR/JAK 2 gene) that could be plays a central role in the induction of myeloproliferative disease in this patient.

Normal and Pathological Erythropoiesis

P034

UNRAVELING THE BIOLOGY OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA DURING ANTI-COMPLEMENT THERAPY: C3-RBC COATING AND EXTRAVASCULAR HEMOLYSIS

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Eculizumab (Soliris®, EC) is a terminal complement inhibitor which proved to be very effective in reducing transfusion requirement and anemia in PNH patients; however, why some patients remain anemic even when intravascular hemolysis is blocked is still an unsolved riddle. To investigate the reasons for suboptimal response to EC, we have studied 35 transfusion-dependent PNH patients receiving EC. All patients showed blockade of intravascular hemolysis, pointed out by a striking LDH level reduction; as expected, this led to cessation (80%) or reduction (20%) in transfusion requirement, but Hb normalization (>11) only in 40%. We had shown a positive C3d Coombs' test in some PNH patients on EC; thus, we systematically investigated C3 coating on RBC, WBC and Plt by flow cytometry, using anti-C3 polyclonal Abs in combination with an anti-CD59 and lineage-specific Abs. Blood cells from healthy controls were always C3 negative, with the exception of neutrophils, which showed a small proportion of $C3^+$ cells in some controls. Untreated PNH patients (n=15) had both $CD59^+/C3^-$ and $CD59^-/C3^-$ but not $C3^+$ RBCs. During EC treatment all 35 patients showed a substantial proportion of $CD59^-/C3^+$ RBCs but never $CD59^+/C3^+$ RBCs; C3 opsonization was first demonstrated one week after therapy, then progressively increasing. Similarly, the same patients showed a consistent C3 positivity on their neutrophils, which was not restricted to PNH ($CD59^-$) cells. In contrast, C3 positivity on Plt was small and erratic. We hypothesized that C3-coating of RBCs due to the lack of CD55 may lead to residual extravascular hemolysis through the reticulo-endothelial system via complement receptors; this is an new mechanism of disease in PNH, which may became apparent only when intravascular hemolysis is blocked. C3 on neutrophils seems to not be linked to the PNH phenotype, and possibly is related to a scavenger mechanism. As proof of principle, one patient still requiring transfusions had Hb normalization after splenectomy. In conclusion, we confirm that EC is the first safe and effective treatment for PNH; we also demonstrate that C3 accumulation on RBC surface is a common finding during EC therapy, possibly leading to residual extravascular hemolysis. Patient-specific propensity to such undescribed form of hemolysis may lie on genetically-determined regulation of complement activation and is currently under investigation.

C035

IN VIVO AND *IN VITRO* EFFECT OF RIBAVIRIN AND INTERFERON ON ERYTHROPOIESIS IN PATIENTS WITH HEPATITIS C

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The combination therapy with Pegylated-Interferon (PEG-IFN) plus Ribavirin (RBV) is the standard of care in patients with chronic hepatitis C. The major side effect of RBV is a mild, reversible haemolytic anaemia. The aim of this study was to evaluate the incidence of haemolytic anaemia in patients treated with RBV and PEG-IFN and to assess the mechanisms underlying anaemia. *In vivo*, we studied 18 patients with chronic hepatitis C treated with a combination of RBV (800 milligrams/die) and PEG-IFN Alpha2a (180 micrograms once weekly) for

24 weeks. All the patients showed a significantly reduction in haemoglobin levels after 4 weeks of treatment (starting point: 14,8±1.1 grams/decilitre; week 4: 12,96±1.5 grams/decilitre). Only 3 out of 18 patients (16%) developed haemolytic anaemia, documented by a marked increase in reticulocytes (3,4%) and in LDH levels (502 Units/liter) and by a significantly decrease in haptoglobin levels (40,3 milligrams/decilitre). To understand the mechanisms underlying anaemia, we evaluated ex vivo the effect of the therapy on erythropoiesis, analyzing the number of patients' pheripheral BFUe along treatment. In 15 patients without signs of haemolysis, a significant reduction in BFUe number associated with a marked increase in undifferentiated CFU-GEMM colony was observed. This suggested that the PEG-IFN plus RBV treatment has an inhibitory effect on erythroid differentiation, supported also by a decrease in fully erythroid differentiated cells and by increased expression of primitive erythropoiesis specific genes (gamma-globin and GATA2). The 3 patients with haemolytic anaemia, on the contrary, had a significant increase in BFUe number after 4 weeks of therapy, suggesting a bone-marrow attempt to compensate the pheripheral haemolysis. *In vitro*, we evaluated the effects of RBV and PEG-IFN on erythroid differentiation of CD34 pheripheral blood cells. Erythroid differentiation was significantly reduced in the presence of PEG-IFN and even more with the combination of PEG-IFN and RBV. All these observations suggest that PEG-IFN and RBV both have an inhibitory effect on erythroid differentiation and are responsible, at least in part, for the anaemia observed in patients during combination therapy. The haemolysis so far attributed to RBV is not the major mechanism underlying anaemia.

P036

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA CASE COLLECTION: LEARNING FROM UNEXPECTED FINDINGS DURING ECULIZUMAB

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Eculizumab (EC) is a mAb against C5 which controls the intravascular hemolysis of PNH caused by from the complement inhibitors (CD55 and CD59) deficiency. EC is the first drug with proven efficacy in PNH, improving anemia, transfusion need, thrombotic risk and QoL. Here we report some findings in 4 patients receiving the standard EC treatment. Patient 1 is a typical hemolytic PNH, who still required transfusions despite LDH normalization by EC treatment. We demonstrated reduced RBC half-life by *in vivo* 51Cr labelling, with excess counts on the spleen, and massive C3 coating on PNH RBC by flow cytometry (with a positive Coombs' test). We hypothesized residual extravascular hemolysis, and decided to remove the spleen; one year after splenectomy, Hb is stably >11 without any transfusion. Patient 2 discontinued effective EC treatment after 2 years due to pregnancy; however, the foetus had been exposed to EC for the first three months. The women received intensive transfusional support and prophylactic heparin without any complication, and had elective caesarean delivery. The baby was born at 36 weeks perfectly healthy, weight 2150 gr, normal APGAR score; she is now 10 months old with a normal psychosomatic development. Patient 3 is a unique case of PNH arisen in a patient who had received autologous transplantation for NHL, experiencing a poor engraftment; flow cytometry revealed a large PNH clone in each lineage (RBC 61%, granulocyte 83%, monocyte 75%). After three months of EC, we observed a contraction of the PNH clone (RBC 12%, granulocyte 7%, monocyte 24%), contrasting with the PNH RBC clone enlargement typically seen during EC. Patient 4 had a history of thymoma associated with myasthenia gravis, poorly controlled pharmacologically; a few years later she also developed PNH. Starting EC, there was a dramatic improvement of her symptoms, including intravascular hemolysis signs and muscular dysfunction. Twelve days after EC administration hemolysis and myasthenia tended to reappear; such a worsening was avoided by reducing the interval from 14 to 12 days, further confirming the benefit of C5 inhibition on both diseases. These observations, while confirming the efficacy of EC in PNH, suggest that: i. residual extravascular hemolysis on EC may require specific treatment; ii. EC may not be contraindicated in case of pregnancy in PNH; iii. EC does impair a possible expansion of the normal hematopoiesis; iv. myasthenia gravis may benefit from EC treatment.

P037

EARLY T CELL RECOVERY AND NK CELLS IN PEDIATRICS PATIENTS AFTER T-CELL DEPLETED HLA-HAPLOIDENTICAL STEM CELL TRANSPLANTATION FOR THALASSEMIA

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Background. Delayed immune recovery post transplant remains a significant obstacle and results in increased risk of infections. T cells are regenerated via 2 pathway, thymus-derived and peripheral expansion, processes for which IL-7 is critical. *Methods*. To analyse the mechanisms involved in immunological reconstitution, we studied six thalassemia patients after 20 and 60 days post transplant. We analysed T cell subsets by flow cytometry, the stromal cells and the stromal IL-7 production by ELISA. *Results.* Day + 20 post transplant, the patients had significantly lower $CD4^+$ T cells in comparison to the controls (1.9±1.4% *vs.* 47.5±6% respectively), and this reduced number was mainly observed in $CD45RA^+CD62L^+$ (naïve phenotype) subset (1.3±2% in patients *vs.* 52±12% in controls). A significant decrease of peripheral $CD45RA^+CD31^+$ Th cells (thymic naive Th cells) (on average 0.5±0.3% in patients *vs*. 37±10% in controls) was observed, whereas $CD8^+$ T cells numbers did not statistically differ between patients and controls (24.2±33.7% *vs.* 20±7%). NK cells were among the first lymphocytes to repopulate the peripheral blood, and up to 70% of these cells were $CD56^{(bright)}$ whereas $CD56^{(dim)}CD16^+$ NK cells were reduced. Day + 60 post transplant an increase in the percentages of $CD4^+$ T cells, naïve $CD4^+$ cells and in thymic naïve Th cells were observed (3±1.2%, 2.9±2.1%, 2.7±1%, respectively). $CD8^+$ T cells were also increased (in mean 35±27.5%).Thalassemia patients showed a significant increase of CD4+ cell activation markers (CD95, HLA-DR and CCR5) and this was observed after 60 days post transplant, in parallel with the increase of the $CD56^{(dim)}CD16^+$ NK cells especially in the patients with full engraftment. Stromal cells secreted lower IL-7 levels (0.3±0.1 pg/mL *vs.* 0.8±0.1 pg/mL, in controls) and displayed by immunohistochemistry an altered phenotype (macrophage-like morphology). *Discussion*. A significant decrease in total lymphocyte counts and depletion of $CD4^+$ T cells expressing predominantly the naïve phenotype were observed after 60 days post transplant. NK cells were among the earliest lymphocytes, but. $CD56^{(dim)}CD16^+$ NK cells were increased after 60 days post transplant, especially in the patients with full engraftment. We hypothesize that the recovery of T cell compartment may be due to a deregulated production of new T cells starting from haematopoietic stem cells under the influence of stromal cytokines production.

P038

ATTENUATED DOSES OF RITUXIMAB FOR THE TREATMENT OF ADULTS WITH AUTOIMMUNE CYTOPENIAS

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A new paradigm of treatment for autoimmune cytopenias has recently emerged, based on the use of monoclonal antibodies such as rituximab. The mechanism by which rituximab reduces erythrocyte and platelet loss in autoimmune hemolytic anemia (AIHA) and in idiopathic thrombocytopenic purpura (ITP), has not been clarified yet. Rituximab is generally infused at 375 mg/sqm weekly for 4 consecutive weeks according to the schedules currently used for the treatment of non-Hodgkin's lymphoma (NHL), in which a high tumor burden is usually seen at diagnosis. However, the number of B lymphocytes in autoimmune disorders is considered normal. For this reason, some attempts have been made using low dose rituximab to treat autoimmune cytopenias in adults. On this basis, 6 patients (4 M; 2 F; mean age 54 years; range 41-66) with autoimmune cytopenias were treated at our Institution with low doses of rituximab (100 mg) for 4 times weekly, after informed written consent was given. Four patients had ITP, 1 patient had warm-antibody AIHA, and 1 patient showed primary cold agglutinin disease (CAD). The median duration of the disease before administer-

ing rituximab was 44 months (range 1-144), whilst the median number of previous therapies was 1 (range 1-3). All patients stopped steroids just before receiving the first rituximab infusion. Five patients completed the 4 scheduled cycles. One patient with ITP did not respond to therapy and rituximab was discontinued after the third infusion. He successfully underwent splenectomy. For all patients, infusion-related side effects were minimal. Complete response (CR) was noted in 2 (50%) of the 4 patients with ITP, and also in both (100%) patients with hemolytic anemia, despite the DAT was still found positive. Three patients with ITP showed an early increase of platelets after the first rituximab infusion followed by a progressive decrease and were considered non responders. The time of maximum response ranged from 2 to 4 weeks. In all responding patients the remission status was maintained at the last follow-up (median 6,8 months; range 2-11) and no steroid therapy was given. Serum Ig levels were detected in some patients and no relevant decrease was observed. Despite the number of patients treated so far with attenuated doses of rituximab is too small to draw any firm conclusion, the results are encouraging and support this therapeutic approach, that however, needs to be validated on a larger group of patients.

P039

PROTOCOL 26 FOR TRANSPLANTATION IN CLASS 3 THALASSEMIA

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Rejection remains a significant cause of transplant failure in thalassemic patients (pts). The outcome of bone marrow transplantation is influenced by the pre-transplant class of risk, being the class 3 (defined by irregular chelation plus hepatomegaly and liver fibrosis, according to Pesaro classification) the one with the worst prognosis due to a more extensive organ damage. The standard conditioning regimens appeared too toxic in these pts. In fact class 3 younger pts (age<17y.o.) prepared for transplant as class 1 and 2 pts (Busulfan [BU] 14 mg/kg plus Cyclophosphamide [CY] 200 mg/kg) showed a 53% probability of thalassemia-free survival and a 39% of transplant related mortality. In an attempt to overcome excessive toxicity, the dosage of CY had been progressively decreased down to 120 mg/kg with a positive impact on mortality (reduced to 18%) but with a concomitant increase in rejection rate, up to 30%. Assuming that BU$^+$ low doses CY was inadequate to suppress thalassemic hematopoiesis, in April 1997 a new regimen (named Protocol 26) has been designed for class 3 young pts in an attempt to improve these results. This protocol involves an intensified pre-conditioning phase with daily azathioprine (3 mg/kg) and hydroxiurea (30 mg/kg) from day -45 through -11, fludarabine 20 mg/m^2 from day -17 through -13 followed by the proper conditioning with BU 14 mg/kg total dose and CY 160 mg/kg (or 90 mg/kg for pts older than 17 y.o.) from day -9 through -2. During the pre-conditioning phase pts undergo a hypertransfusive regimen to keep Hb higher than 14 gr/dL, a twice a week administration of growth factors (Erythropoietin and G-CSF) and a continuos 24h daily infusion of desferoxamine in CVC. The first 33 class 3 pts (median age 11 y.o., range 4-16) treated in Pesaro with protocol 26 obtained survival, thalassemia-free survival, rejection and non rejection mortality rates of 93%, 85%, 8% and 6% respectively. From June 2004 through August 2007 35 class 3 pts (median age 10 y.o., range 5-16) have been transplanted with the protocol unmodified in Rome at the International Centre for Transplantation in Thalassemia and Sickle Cell Anemia. The survival, thalassemia-free survival, rejection and non rejection mortality rates were 87%, 80%, 12% and 10% respectively. The Rome experience confirms the good results obtained in Pesaro, the efficacy of this protocol in reducing graft rejection in such compromised pts without an increased mortality and its reproducibility.

P040

HB ERNZ [BETA123(H1)THR->ASN]: A CASE REPORT

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Introduction. More than 750 hemoglobin variants have been described; some of them have been discovered because they have electrophoretic properties differing those of HbA. Analysis of globin chains by reverse phase high performance liquid chromatography (HPLC) and genetic sequencing are additional tools for typing some hemoglobin variants that are different from HbA for hydrophobicity and not diagnosticable with common electrophoretic technique (cellulose acetate electrophoresis at alkaline pH and with cation exchange HPLC). The Hb Ernz [beta123(H1)Thr->Asn], a very rare hemoglobin variant, has been described for first time by Groff P et all in an Italian subject; here we described the second case in literature of Hb Ernz. *Materials and Methods*. Hematological studies have been performed by routine methods on a Cell-Dyn 3700 counter (Abbott Labs). Electrophoretic analysis of Hb has been performed on cellulose acetate electrophoresis at alkaline pH. Chromatographic Hb analysis has been determined by Cationic Exchange HPLC (beta-thalassemia dual kit - Biorad VARIANT 2). Globin chains analysis has been performed by reverse phase high performance liquid chromatography (RP HPLC) on Biorad VARIANT using Lichrospher 100 RP 8 column Merck. Beta globin gene mutation study has been carried out with Reverse Dot Blot (Nuclear Laser Medicine kit lab) and Genic sequencing according Sanger on a ABI System 310 (Applied Byosistem). *Case Report*. A 28 years old patient of Apulian origin (Italian), partner of a heterozigous beta talassemic female pregnant at 9th gestational week, has been studied. Hematological data were: RBC 5.9×10^{12}/L, Hb 15 g/dL, MCV 73.6 fl, MCH 25 pg, WBC 8.5×10^9/L and PLT 208×10^9/L. Cationic Exchange HPLC showed HbA2 3.3% and absence of any Hb variant. No Hb variant has been showed on cellulose acetate electrophoresis at alkaline pH. Beta gene molecular study has been performed for suspect of a mild beta gene mutation; any Italian common mutations have been detected. Beta gene sequencing, showed a nucleotidic substitution (C→A) in heterozigous of 123 codon of 3rd exon corrisponding at substitution Thr→Asn. Abnormal beta chain has been confirmed by RP HPLC and amounting to 40% of total beta chains. The finding of alpha$^+$ mutation of alpha globin gene (alpha -3.7kb) is responsible for low MCV. In literature is not reported the hematological clinical phenotype of Hb Ernz and beta talassemia, therefore our case showed off prenatal diagnosis problems.

P041

EVIDENCE FOR REDUCED ANGIOGENESIS IN BONE MARROW IN SYSTEMIC SCLEROSIS

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Background. Dysfunctional angiogenesis is a pathogenetic marker of SSc. Microvascular endothelial cells have a reduced capacity to form capillaries (1), reduced circulating levels of endothelial progenitor cells have been found (2) and mesenchymal stromal cells differentiated in endothelial cells have shown a defective capacity to form capillaries (3).The hypothesis is that some modifications are in act already in the BM of SSc patients. *Objectives*. To study in SSc BM the angiogenetic process, the cellular immune system and fibrosis. *Methods.* 8 SSc patients affected by a severe diffuse SSc and screened for autologous hemopoietic stem cells transplantation, underwent a BM biopsy to assess cellularity and morphology. BM biopsies were compared with 5 healty controls. To evaluate angiogenesis, immune system and fibrosis the following antibodies were used: VEGF, KDR/flk-1, MMP-9, CD34/QBEND10, vWF, CD20, CD3, CD4, CD8, CD38, K, lambda, CD68/PGM-1, CD61. To evaluate fibrosis silver impegnation for reticulum was used. The number of vessels, the mean area of vascularisation, the perimeter and microvessel density (MVD) were measured with a multiparametric computerized imagine analysis. *Results*. Morphology of BM was similar in SSc and controls. Also B cells population was similar but only a reduction of CD4/CD8 ratio was observed in SSc. A significant reduction in BM vascularity was found: both microvessel density and number of vessels were lower while VEGF expression was much higher than in controls. In seven patients a weak expression of KDR/flk-1 was observed and MMP-9 expression was low in all cases.Out of 8 patients 2 had a maximal while other 2 had a moderate grade of BM fibrosis. *Conclusions.* In SSc,BM is characterised by a reduction of angiogenesis that may induce the increase of VEGF.

P042

THROMBOTIC MICROANGIOPATHY: CLINICAL, DIAGNOSTICS AND THERAPEUTIC CHARACTERISTICS. ANALISYS OF SIX CASES

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Introduction and study objective. Thrombotic microangiopathy (TMA) is a clinicopathological syndrome characterized by microthrombi in the arterioles and capillaries of many organs. TMA included haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). TTP is a disseminated thrombotic microangiopathy that mainly appears as idiopatyc disease, but sometimes is secondary to different disease. Clinical characteristics are: haemolytic anemia, thrombocytopenia with hemorrhagic manifestation, neurological focality and renal impairement. We analized in a retrospective way six patients (pts) affected by TTP during the last 28 months between December 2005 and April 2008 in the Department of Haematology and Transfusion Medicin of our institution. This study aimed to evaluate clinical and laborathoristics parameters related to therapeutic approach. *Results.* In the last 28 months, six cases were diagnosed. Mean age was 44,8 years; 5 female and 1 male. Three pts had showed neurological symptoms such as headaches, focal manistation, convulsions. Fever of unknown origin was evident in one patient (pt) while gram positive bacteremia was documented in two pts and impairement of renal function in one. Hemorrhagic diseas was documentated in two pts (skin localization). TTP pathogenesis was: idiopathic in three pts; other three pts had respectively: associated to metastatic carcinoma, recurrent idiopathic and pregnancy related. At diagnosis, medium hemoglobin level was 8.36 g/dL (7.1-11.5), reticolocytes 8.9% (2.5-17.6), platelets $44\times10^3/mm^3$ (5-128) LDH 2068 UI/L (641-4378), Aptoglobin 10 (5-29); schistocyte 10/field. All patients received five continuative plasmapheresis (pex) with CS3000 Fenwal device. Mean plasma volume processed was 2000 mL (range 1800-2000) by mean flow rate of 60 mL/min. During all procedure pts received fresh frozen plasma (rate 1:1). Subsequent pex (7-21 pex – median of 10 pex/pt) was done every two days monitoring clinical and haematological paramethers. All pts received supportive therapy by prednisone (1 mg/kg/die) and ASA (100 mg/die). Elevated levels of AST, ALT and bilirubinemia was documented in pregnancy patient with diagnosis of AFLP (acute fatty liver of pregnancy). At this time no relapses was documented at mean follow up of 13 months. A exitus was documented in the pt with TTP associated to metastatic carcinoma. *Conclusion.* A multidisciplinary approach, a rapid diagnosis and a prompt debut of plasma-exchange is really neaded in TTP patients. Diagnosis of TTP is not so rare than before and prognosis is now better, due to optimization of the plasma-exchange schedule and supportive therapy with steroid and platelet antiaggregants drugs. Maintenaince treatment protocol and adeguate follow-up can minimize the relapse rate.

P043

SUCCESSFUL USE OF ECULIZUMAB IN A VERY RARE CASE OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA FOLLOWING STEM CELL AUTO TRANSPLANTATION

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Paroxysmal nocturnal hemoglobinuria (PNH) is an uncommon haemolytic anemia, caused by a clonal expansion of hematopoietic stem cells that harbor a mutation in the phosphatidylinositolglycan complementation Class A (PIG-A) gene, leading to a deficiency of GPI-anchored proteins (GPI-APs). The onset of PNH after auto stem cell transplantation (ASCT) for non-Hodgkin lymphoma (NHL) has never been reported. A 52-year-old man was admitted for stage IV A (BM^+), FLIPI 2, grade III NHL, received R-CHOP chemotherapy, obtaining a CR. After 5 months he relapsed, so started the MIMA regimen, followed by HD sequential chemotherapy with VP16, EDX, ARA-C and rituximab. After ASCT, persistence of mild pancytopenia was observed; biopsies showed multilineage dysplasia. After 11 months he complained increasing astenia, tachycardia, esophageal spasms and dark urine. Laboratory data showed pancytopenia, reticulocytosis, elevated LDH and liver enzymes; haptoglobin was reduced, Coombs test negative, urine analysis highly positive for blood without red cells in sediment and intense hemosiderinuria; the BM trephine biopsy and 18FDG-PET confirmed the CR of NHL. Flow cytometry with anti CD59/CD55 showed a large red cell population with undetectable expression of GPI-APs; similar results were obtained in granulocytes and monocytes (anti CD16-CD14). PNH diagnosis was made; prednisone was started, with an unsatisfactory control of the periodic hemolytic crisis; worsening clinical condition occurred, with weekly blood transfusion. Then the patient started therapy with the terminal complement inhibitor eculizumab (EC). Hemolysis rapidly decreased, the patiens became transfusion-independent, and in a few weeks Hb levels stabilized at about 11.5 g/dL. EC was well tolerated; no episodes of hemoglobinuria, dysphagia, headache or abdominal pain occurred, the quality of life improving significantly. Some reports indicate that patients receiving ASCT may have increased risk for MDS-AML. In this unusual case, the onset of PNH was not linked to a relapse of NHL; instead it was preceeded by trilinear dysplasia that finally evolved into clonal expansion of PIG-A mutated stem cells. We performed cytometric analysis of the criopreserved cells obtained during PBSC mobilization before ASCT but we found that there was normal CD59/CD16 expression, so excluding a latent PNH before ASCT. Even in this rare case EC significantly reduced hemolysis resulting in improved anemia, fatigue, quality of life.

P044

PERIPHERALLY INSERTED CENTRAL CATHETERS AND MIDLINE CATHETERS AS ALTERNATIVE VASCULAR DEVICES IN CHILDREN SUBMITTED TO HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR THALASSEMIA

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Thalassemia is an inherited bone marrow disease requiring chronic long-life transfusions to treat the anemia caused by enhanced red blood cell destruction; this leads to progressive iron overload followed by organs deterioration. The only radical cure is haematopoietic stem cell transplantation (HSCT), which can give a very high rate of survival and disease-free life expectancy. To do this, patients submitted to HSCT need a specific central vascular access where all intravenous drugs and blood samples are given. In our institution, we perform at least six allogeneic bone marrow transplants monthly, most of all in pediatric patients, coming from the Mediterranean areas in which thalassemia and some other haemoglobinopathies are endemic; given this, there is suggestion for choosing the best catheter to be used for those patients, with a wide variety of vascular devices which can improve the care and management of the patient himself, particularly his quality of life, and which may support the best practice for nurses and physicians. To date, the review of the literature shows the benefits given by ultrasound technique for the positioning of a central venous catheter, which can be inserted also by peripheral access, and illustrates how this procedure can be performed by anyone who has the specific know-how. These devices, named PICC (Peripherally Inserted Central Catheters) and MIDLINE catheters, could ensure a valid access for patients who are under treatment during pre and post-transplant period; they are also progressively becoming an alternative choice in the use of traditional tunnelled catheters, but there aren't still enough data for this kind of patients in the literature. Our experience started on February 2008 to evaluate the efficacy, referring to best quality of life, the expected lower rate of infections and replacements of the above mentioned devices, and the cost-effectiveness. For this reasons we decided to adopt a new strategy in administrating drugs, blood and platelets transfusions, parenteral nutritions, in performing blood samples both in charged and discharged patients, to observe if these devices give advantages in the management of these pediatric patients waiting for HSCT. We hope that the use of these devices will carry on advantages to improve care, management and quality of life of the patients, supposing evidences could come from easy availability of ultrasound technique, easy possibility to perform the insertion bed-side, respecting all warnings in terms of patient's and operator's security, and easy acquisition of the specific required know-how, with the aim to reduce waiting time for the insertion.

P045

IMMUNOPHENOTYPING OF WEAK AND PARTIAL RH(D) ANTIGEN: OUR EXPERIENCE

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Background. The D antigen of the Rh system is a mosaic of epitopes. In Europe, about 1% of people carries Rh(D) alleles as weak or partial D. A weak D type results from a quantitative reduction of the Rh(D) antigen, whereas a partial D type indicates a qualitatively altered Rh(D) protein. Individuals, whose cells lack some epitopes of the D mosaic, may make antibody to these epitopes when exposed to a complete D antigen. Many studies have shown that there are more than 30 recognisable epitopes, but the 9 epitope model is clinically satisfactory. With the use of selected panels of monoclonal anti-D reagents it is possible to distinguish the red cell categories of partial-D phenotypes. Based on different serological properties from a weak D type, a partial D type was suspected in cases with anti-D in their serum or if nonreactive to some reagents. In this study we have examined the reactivity patterns of red blood cells, resulted non reactive to routinely used anti-sera, with 6 different monoclonal anti-D able to obtain an accurate characterization of Rh antigen. *Materials and Methods*. We collected a total of 52 samples from blood donors and patients screened as Rh-negative in RhD typing with monoclonal and polyclonal anti-D between 2003 and 2007. Further, we performed RhD typing with a column agglutination test, weak D by the standard tube test and finally partial D using the ID-partial RhD-typing, a card with 6 microtubes containing polyspecific anti-human globulin within the gel matrix. (Diamed, Switzerland). The sera can differentiate between categories II, IV, V, VI, VII, DFR, DBT, R0Har. Category I is obsolete and category III red cells react with all anti-D reagents but can be distinguished from normal Rh D positive cells by the presence of allo anti-D in the serum. In Table 1 are represented reactions for partial Rh D characterization. *Results*. The D tube test showed D weak in 41 of 52 blood samples tested. 1 was genotypized as D type 3 (associated to IAT positive for anti-D), 7 as type V, 1 as type VI (associated to Ce) and 2 type VII (associated to Ce). *Conclusions.* Considering that the most partial D discovered in the Caucasian population was weak D, it is recommended that Rh(D) typing and/or weak D tests in blood donors should be done using more than two anti-D reagents from different clones. Our survey is reported in the hope that it may find some use as reference for studies of blood group systems and indicates as many others studies that molecular classification of weak D offers a more reliable basis than serotyping and is relevant to optimal D transfusion strategies. Partial DVI remains the most important category to define. Clinically, patients with partial DVI red cells should be treated in the same way as Rh(D) negative individuals both for transfusion purposes an during/after pregnancy with a Rh D positive fetus, to prevent the alloimmunization versus the missing epitopes with possible subsequent clinical sequelae.

Table 1.

	Expected reactivity for partial D									
Anti-D	*DII*	*DIII*	*DIVa*	*DIVb*	*DV*	*DVI*	*DVII*	*DFR*	*DBT*	*R0Har*
1	+	+	-	-	+	+	+	+	-	-
2	-	+	-	-	+	+	+	+	-	-
3	+	+	-	-	-	-	+	-	-	-
4	+	+	+	+	+	-	-	-	+	-
5	+	+	+	+	+	-	+	+	-	-
6	+	+	+	+	+	-	+	-	+	+/-

P046

TREATMENT OF ANEMIC PATIENTS AFFECTED BY HYPERFERRITINEMIA BY PHLEBOTHOMY AND ERITROPOIETIN

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Background. Hyperferritinemia is a disease characterized by a primitive pathogenesis such as Hereditary Haemochromatosis (HH). More frequently, it is secondary caused by different types of pathologies such as: transfusions or administration of intra venous/oral iron, anaemia with deficient haematopoiesis. The administration of the drugs such as Desferroxamina is a therapy complementary to the only treatment still assented: the phlebotomy. On the other hand, these therapeutic treatment are often interfered by low levels of Haemoglobin (Hb) and/or Haematocrit (Ht) values. *Aims.* The aim of this study was to evaluate the efficacy of the therapeutic association of phlebotomy and Eritropoietin (EPO) in the treatment of patient affected by hyperferritinemia to prevent the premature anaemia. *Methods and Results.* We have studied 2 patients affected by hyperferritinemia and negative for the HFE, TFR2 and FFN1 mutations genes, submitting them to cycles of phlebotomy accompanied by administrations of EPO 40.000 UI s.s. The first patient, a 60-year-old man, showed a high serum ferritin value of 1.869 ng/mL. Furthermore, the patient was affected by chronic hepatitis HCV +, and his values of Hb and Ht were respectively of 12,1g /dL and 36,4%. After two phlebotomy he showed Hb 10,2 g/dL and Ht 29,1%. Subsequently, we have administered EPO 40.000 UI s.s. followed by a phlebotomy after 7 days and contemporary administration of EPO 40.000 UI. After 4 phlebotomy the patient achieved a ferritn value < 50 ng/mL, Hb 12,1 g/dL, Ht 34%. The second patient, a 56-year-old man showed a high serum ferritin value of 693 ng/mL and he resulted negative for the HFE, TFR2 and FFN1 mutations genes. Besides, the patient showed a MCV of 69 μm^3, Hb 11,4 g/dL, Ht 36,5%, osmotic iper-resistance and increased levels of the Hb A2. The patient was submitted to the same treatment above described (EPO 40.000 UI s.s/ 4sessions). At the end of the cycle the serum ferritin final results were 210 ng/mL and it was shown Hb12,1 g/dL and Ht 39%. *Conclusion*. Our study suggests that the association of phlebotomy and EPO results to be an effective treatment for secondary hyperferritinemia, negative for the HFE, TFR2 and FFN1 mutations genes. In fact, this therapy has allowed us to achieve in rapid times a large reduction of the serum ferritin values, because of a greatly mobilization of the iron by the pool of deposit to the circulating pool, with persistence of values of Hb and Ht in the normal range.

P047

A CASE OF CHILDHOOD PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA: SAFETY AND EFFICACY OF THE TERMINAL COMPLEMENT INHIBITOR ECULIZUMAB

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Paroxysmal nocturnal hemoglobinuria (PNH) is a rare disease caused by a somatic mutation of the PIGA gene, which results in the absence of the glycosylphosphatidylinositol anchored proteins necessary to protect cells from complement-mediated lysis. The clinical manifestations of PNH include intravascular hemolysis, fatigue, thrombosis, poor quality of life, transfusion dependency and bone marrow failure. Onset usually occurs in adulthood. Few children and adolescents with PNH have been described and laboratory data on diagnosis, clinical course, survival and therapy in young patients are unavailable. Overall results from two Phase III clinical trials show that the terminal complement inhibitor EC significantly reduces hemolysis resulting improvement of anemia, fatigue and quality of life. A 18-year-old girl referred astenia, tachycardia and emission of dark urine. Laboratory data showed severe anemia (Hb 8 g/dL) with reticolocytosis (200.000/microL), lactate dehydrogenase about 2200 U/L and reduced seric haptoglobin (<0.07 g/L). The young girl presented periodic hemolytic crisis with worsering quality of life while needing blood transfusions. Flow cytometry analysis with anti CD59/CD55/CD66b antibodies demonstred a large cellular population of granulocytes (98%) and erythrocytes (87%) with no detectable expression of GPI-anchored proteins. So a PNH diagnosed has been made; given the transfusion dependence, a treatment by eculizumab was considered. The patient was vaccinated against Neisseria meningitidis. Then, she received infusion of EC as follows: 600 mg intra venous every 7 days for four weeks, 900 mg one week later and then 900 mg every 14 days for a total of 21 weeks of therapy. Clinical and biochemical indicators of hemolysis were measured weekly. After starting eculizumab therapy, hemolysis rapidly improved as shown by a 78% decrease of lactate dehydrogenase (LDH) levels (from a median of 2130 U/L before treatment to 440 U/L at 21 weeks). The patient became transfusion-independent, and Hb levels increased from baseline stabilized at 11 g/dL in the absence of transfusions. Eculizumab was well tolerated. Throughout therapy no episodes of hemoglobinuria, dysphagia or headache were recorded and the quality of life improved significantly.This report suggests that EC treatment may be extended to younger patients; however, further larger studies are needed to test the safety and efficacy of EC in children.

Chronic Myeloid Leukemia and Myeloproliferative Disorders (I)

P048

A PROSPECTIVE RANDOMIZED STUDY OF IMATINIB 400 MG VS 800 MG AS A FRONT-LINE THERAPY IN HIGH SOKAL RISK PH-POSITIVE CHRONIC MYELOID LEUKEMIA PATIENTS IN CHRONIC PHASE

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Imatinib (IM), 400 mg daily, is the drug of choice for the front-line treatment of Ph-pos chronic myeloid leukaemia (CML). Several biologic findings and clinical observations suggest that increasing the dose of IM may result in improved efficacy. The complete cytogenetic response (CCgR) rate to IM 400 mg is significantly affected by Sokal risk (49% in high risk - HR - patients at 1 year, IRIS study). Thus, newly diagnosed HR patients may benefit from a dose increase front-line. To compare the effects of IM 400 mg or 800 mg daily in previously untreated, early chronic phase, Sokal HR, Ph pos CML patients was planned an international (Italy, Scandinavian countries and Turkey), multicenter, prospective, randomized study: 215 patients were enrolled and randomized (1:1) to receive IM 400 or 800 mg daily. Cytogenetic response was assessed by chromosome banding and FISH analysis of marrow cells at 3, 6 and 12 months. Molecular response was assessed by Q-PCR of blood cells at 3, 6, and 12 months. The primary efficacy variable was the CCgR rate at 12 months, based on the intention-to-treat. As of February 2008, 205/215 patients are evaluable for the primary efficacy variable, 103 in the 400 mg arm, and 102 in the 800 mg arm. The CCgR rates (400 mg *vs.* 800 mg) were 20% *vs.* 23% at 3 months, 53% *vs.* 52% at 6 months, and 60% *vs.* 63% at 12 months. Treatment failures were 19/103 (18%) in the 400 mg arm *vs.* 16/102 (16%) in the 800 mg arm. In the 400 mg arm, the median administered dose was 400 mg, and there was no difference in outcome between the patients who received the full dose or a median dose ranging between 300 to 400 mg. In the 800 mg arm, the median administered dose was 700 mg, and the failure rate was lower in those who received a median dose between 700 to 800 mg than in the others. Treatment discontinuations for adverse events were 4/103 in the 400 mg arm *vs.* 7/102 in the 800 mg arm. Molecular response data are under evaluation and will be presented on site. In conclusion, in this international, prospective, randomized study of IM 400 *vs.* 800 mg daily in a selected, high risk, population of patients with Ph-pos CML, no difference could be detected in the rates of CgR, failure and adverse events during the first 12 months of treatment. The patients will be followed for progression-free and overall survival. Acknowledgements: European LeukemiaNet, Regional Oncology Centre of Uppsala, COFIN, University of Bologna and BolognAIL.

P049

COMPARATIVE EVALUATION OF REAL-TIME PCR ASSAYS FOR JAK2V617F MUTATION: STEP TOWARDS ASSAY HARMONIZATION

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The discovery of JAK2V617F mutation has represented a tremendous advance in the diagnosis and classification of Phildelphia-negative chron-

ic myeloproliferative disorders (MPD); in fact, presence of the mutation represents a major diagnostic criterion. There is also growing evidence that the amount of mutated allele (V617F allele burden) is associated with significant clinical endpoints. These considerations make the availability of sensitive and reproducible quantitative assays for the V617F allele a fundamental requirement for diagnosis and prognostication; as a matter of fact, there is a myriad of published methods that employ different approaches, primer and probe design, reference genetic material, and also use different cell sources, and reproducibility of results among different labs is unknown. As a preliminary step toward assay harmonization, we have comparatively evaluated five different real-time based PCR assays, published in major journals, that differed as concerned characteristics of mutation-specific primers or probes. There were two assays based on mutation-specific conventional probes (A, B), one each differing for mutation-specific LNA-modified probe (C), mutation-specific reverse primer (D) or mutation-specific forward primer (E). For each of these, we produced standard curves to calculate performance parameters and in particular the sensitivity of the assay. We observed significant differences in discriminatory capacity of the assays, with best performance for assay B and C, in which the DeltaCT discriminatory value from 0 to 100% V617F allele was around 25 cycles, as compared to 14 cycles for assay A and E and 3 cycles for assay D. A 0.1% mutated allele burden could be reliably detected using assay C and E. No healthy subject was erroneously genotyped as mutated. Inter-assay and intra-assay variation coefficients were in the range of 1-3% for all assays, while among-assays coefficient was 2-5% except for assay D (10-12%). We also evaluated in parallel gDNA obtained from granulocytes or whole peripheral blood (PB) of the same subject; in more than 50 patients evaluated, genotyping was concordant but values obtained in PB were 5-7% lower than in granulocytes. These data suggest that published assays and different cell source of gDNA may introduce some heterogeneity, and anticipate the opportunity of actions for standardization of techniques employed for JAK2V617F genotyping and quantification.

P050

ACTIVATED BASOPHILS IN PATIENTS WITH POLYCYTHEMIA VERA, AND RELATIONSHIPS WITH JAK2V617F MUTATION

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Clinical features of polycythemia vera (PV) include pruritus, which is often exacerbated by contact with water and may be the most agonizing aspect of the disease. To investigate possible pathogenetic mechanisms underlying this feature, we studied the activation status of basophils in patients with PV (either suffering or not from pruritus) or essential thrombocythemia (ET), and also in two patients with systemic mastocytosis (SM). We measured by flow cytometry the expression of activation marker CD63 in whole blood samples. Median percentage of activated basophils was: 21.9 (range 21.6-23.3) in PV patients suffering from pruritus as compared to 6.7 (range 1.59-20) in PV patients without pruritus (p=0.00004), 3.7 (range 0-12.6) and 0.75 (range 0.7-0.8) in ET and SM respectively, and 1.8 (range 0-5.4) in healthy subjects. Patients affected by PV with pruritus had also a significantly greater CD63 MFI compared to PV patients without pruritus (mean 2,955 *vs.* 1,372, p=0.004). Since occurrence of pruritus has been found strongly associated with higher JAK2V617F allele burden, we measured V617F allele burden in immunomagnetically purified basophils, and found it to be similar to that measured in granulocytes. However, JAK2 mRNA levels in basophils, measured by real-time PCR, were significantly greater than in neutrophils (p<0.0001). To further investigate the role of JAK2V617F in basophil activation, we studied the expression of mouse mast cell proteases in the IL-3 dependent murine pro B cell line BA/F3, both parental and transfected with the murine wild type, murine V>F and human V>F JAK2 (kindly provided by Dr R. Skoda). We found that the murine tryptase MMCP-6, that was not expressed in the parental line, was strongly induced in cells transfected with JAK2V617F, while mRNA levels of microphthalmia-associated transcription factor (MITF) were unchanged. Light microscopy analysis showed increased number of basophils in PV patients compared to controls. Electron microscopy analysis revealed strongly abnormal morphology of granules, some of which appeared empty, and of granules membrane appearance. These data suggest that activated basophils in the circulation of PV patients might contribute to the pathogenesis of disease-associated pruritus, and underscore a role of mutated JAK2V617F in basophil activation.

P051

A SCORING SYSTEM BASED ON LEUKOCYTE ALKALINE PHOSPHATASE ACTIVITY AND PERIPHERAL GRANULOCYTE PRECURSOR PERCENTAGE FOR PREDICTING JAK2 V617F MUTATION IN PATIENTS WITH PRIMARY MYELOFIBROSIS

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A recurrent mutation in the JAK2 gene consisting of a valine-to-phenylalanine change at position 617 (JAK2 V617F) was reported in half and more of the patients with primary myelofibrosis (PMF). This mutation increases JAK2 kinase activity affecting phenotype and clinical outcome. Recently, some of us observed that in PMF JAK2 V617F mutation is significantly associated with higher white blood cell count and granulocyte activation and it independently predicts the evolution toward large splenomegaly, need of splenectomy and leukemic transformation. Since increased leukocyte alkaline phosphatase (LAP) expression is considered a marker of granulocyte activation and variable LAP levels are found in PMF, we evaluated LAP activity by cytochemistry in peripheral blood smears from 249 PMF patients at diagnosis and searched for possible correlations among LAP score, JAK2 V617F mutation status, as assessed by allele-specific PCR, and clinical-pathological features. JAK2 V617F mutation was identified in 161 patients (64%). In these patients LAP scores were significantly higher than in nonmutated cases (p<0.0001), without difference between homozygous and heterozygous mutations. Score values above the normal range were observed only in patients carrying the mutation. No correlation was found between LAP score and white blood cell or platelet count, hemoglobin concentration, splenomegaly, Lille prognostic score, while there was a significant inverse correlation between LAP score and peripheral granulocyte precursor or blast percentage (p<0.0001). On the other hand, a multivariate analysis showed a significant association of JAK2 V617F mutation with lower peripheral granulocyte precursor percentage. A ROC curve analysis allowed us to identify a LAP score of 100 (AUC=0.822, 95% CI 0.796-0.869) and a peripheral granulocyte precursor percentage of 10 (AUC=0.81, 95% CI 0.755-0.857) as optimal cut-off to discriminate mutated patients with good sensitivity and specificity (range 84-98%). Thus, on the basis of these variables, we defined a simple scoring system to predict JAK2 V617F mutation (Table 1). All cases with a score of 3 carried the mutation, while 94% of patients with a score of 0 showed a wild-type gene. Then, we prospectively tested this score system in a new cohort of PMF patients obtaining superimposable results. In conclusion, we suggest a very simple, low expensive and reproducible method based on old techniques to predict a novel mutation.

Table 1.

Score		
0	*1*	*2*
LAP score <100	-	LAP score ≥100
Peripheral granulocyte precursors ≥10%	Peripheral granulocyte precursors <10%	-

P052

THE ACHIEVEMENT OF A PRECOCIOUS MAJOR MOLECULAR RESPONSE DOES NOT SIGNIFICANTLY CONDITION PROGRESSION-FREE-SURVIVAL: A MOLECULAR MONITORING OF 77 CML CASES

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Imatinib induces a complete cytogenetic response (CCR) in more than 80% of patients affected by chronic myeloid leukemia, with prolonged overall and progression-free survivals (PFS). Nevertheless, about 20% of cases lose CCR; this is the subgroup where the quantitative RT-PCR

monitoring is useful for predicting relapse. Indeed, the attainment of major molecular response (MMR) (BCR-ABL/ABL<0.1%) identifies a subgroup of CCR patients with lower relapse rate. On the other hand, it has been reported that a half-log increase in BCR-ABL mRNA predicts a higher risk of relapse. In this study, we molecularly monitored 77 patients for a median of 48 months, in order to assess if PFS could be predicted by dynamic of BCR-ABL transcript, especially focusing on precocious MMR achievement. Fourteen cases (included in the IRIS trial) received imatinib in late chronic phase, the remaining 63 at diagnosis. From 2006, molecular monitoring was performed according to European Net Guidelines; before, MMR was defined as reduction of transcript higher than 3.5 logs in comparison to basal value, to overcome the variability of BCR-ABL mRNA at diagnosis. Clonal evolution, progression to accelerate phase/blastic crisis, and loss of CCR were considered *events* in the calculation of PFS; 5y-PFS was 66% for the entire series and 72% for cases treated up-front. With a median time of 6 months, 85% of patients achieved CCR that significantly conditioned PFS (median 83 months *vs.* 33 months in cases with minor cytogenetic response, $p<0.01$). After 3 months, 10% of patients achieved MMR; by 24 months, 50% were in MMR. The achievement of MMR significantly conditioned PFS after 18 and 24 months of treatment (median 62 months *vs.* not achieved), without any statistical significance in cases achieving MMR at 3 months. Interestingly, during follow-up 42% of cases showed at least once increased BCR-ABL/ABL ratio; nevertheless, the median increase was 1 log and this phenomenon did not condition the relapse rate. On the contrary, 5y-PFS was significantly conditioned by the dose of imatinib administered; 27% of patients received <400 mg/day for haematological toxicity; in this subgroup, 5y-PFS was 38% *vs.* 78% for patients receiving 400 mg/day ($p=0.02$). In conclusion, our results show that the precocious MMR is not predictive for a longer PFS, the BCR/ABL transcript is often fluctuating, but in a limited range, and particular relevance would have the achievement of adequate imatinib plasma levels.

P053

COMPARISON OF THREE DIFFERENT MOLECULAR TECHNIQUES FOR DETECTION OF JAK2 V617F MUTATION IN CHRONIC MYELOPROLIFERATIVE DISORDERS: ADVANTAGES AND FAULTS

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JAK2 V617F mutation has been reported in >90% of polycytemia vera (PV), 60% of essential thrombocythemia (ET) and 50% of idiopatic myelofibrosis (IMF). In the first phase of the study, we tested three different molecular techniques for mutation detection in order to identify the most sensitive, rapid and the reproducible one. Bone marrow or peripheral blood were obtained from 84 individuals recruited from two different Centres (PV 24,7%, ET 45,5%, IMF 16,9%). In the second phase, we employed the most sensitive technique for evaluating 75 patients affected by chronic Ph'-negative myeloproliferative disorders, 22 cases with secondary polycytemia ot thrombocythemia and 20 healthy controls. Firstly, we employed the automatic DNA sequencing on ABI PRISM® 310. The second technique was a real-time PCR developed for allelic discrimination. Two LNA probes, reporting two different dyes, were designed. Finally, all samples were screened by allele-specific PCR (ARMS PCR). One of the two primers was marked with 6-FAM, allowing products analysis on the automatic DNA sequencer. The percentage of mutated and wild-type alleles was determined by calculating the proportion of the respective peak areas as compared to the sum of both peaks areas. In order to test the sensitivity of different methods, K562 cells were mixed with decreasing proportions of HEL cells carrying JAK2V617F mutation in homozygous state. ARMS PCR showed the highest sensitivity (1:10000) in comparison to the other two techniques. By this method, in 93% of cases affected by PV, 70% of ET, and 60% of IMF JAK2 mutation was detected. The percentages of homozygous cases were 20% in PV, 12% in ET, and 30% in IMF. Surprisingly, even 5 of the 20 healthy controls showed the JAK2 mutation, with mutated allele ranging from the 1.4% and 2.5%. The same samples resulted unmutated when analyzed by real-time PCR. In another case, the mutation amounted to 16%: this patient was screened because of a mesenteric thrombosis. Our data confirm that different techniques are suitable for detecting JAK2 mutation. Nevertheless, we suggest the use of this modified ARMS-PCR that shows higher sensitivity and that offers a quantitative measure of mutated status, potentially useful in the monitoring these patients. Nevertheless, when this method is adopted, attention must be done for cases presenting very low percentage of JAK2 mutation in order to not over-estimate the JAK2 V617F presence.

P054

MARKED TELOMERE EROSION OF PH-NEGATIVE MYELOID CELLS AFTER SUCCESSFUL TREATMENT OF CHRONIC MYELOGENOUS LEUKEMIA INDICATES SEVERE PROLIFERATIVE STRESS AND POTENTIAL PERMANENT DAMAGE OF HEMATOPOIESIS

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Background. Most chronic myelogenous leukemia (CML) patients (pts) experience long-term restoration of non-neoplastic hematopoiesis following successful disease treatment with tyrosine kinase (TK) inhibitors. However little is known on the functional and genetic integrity of Ph-negative cells repopulating the bone marrow after successful disease control. Indeed, they might have suffered considerable stress due to the interaction with the malignant clone and/or the subsequent effort of bone marrow (BM) repopulation. This hypothesis is also suggested by the occurrence of cytogenetic abnormalities (CA) in Ph-negative cells in about 10% of these subjects. *Aims.* Telomere restriction fragment length (TRF-L) analysis is an effective tool to monitor the proliferative stress of hemopoietic cells as shown by several transplantation studies. Aim of this study was to use TRF-L analysis to verify the presence and degree of proliferative stress suffered by the hematopoietic compartment repopulating bone marrow following successful disease control. Patients and methods 53 chronic phase CML pts in complete cytogenetic remission (CR) lasting one year or more were analyzed. 43 pts were treated with Imatinib and 10 pts with INF associated or not to ara-C. Median age was 61 (23-88), M/F ratio was 1.5, median time from diagnosis and from complete CR were 70 (12-217), and 40 months (6-150), respectively. 24 pts had low Sokal score, 19 intermediate, and 10 high. 7 pts carried an acquired CA. Complete molecular responders were 20 of 43. 86 healty donors with a comparable age and M/F were used as controls. TRF-L analysis was performed by Southern Blotting as previously described (Ladetto M *et al.*, Blood 2004), both on peripheral blood (PB) polymorphonucleates (PMN) (isolated as described in Tarella C *et al.*, Eur J of Cancer 1991) and on monocyte depleted PB mononuclear cells (MD-PBMC) (obtained as described by Ferrero D *et al.*, J Clin Lab Imm 1998). This allowed to assess both the myeloid and lymphoid compartment. *Results*. Figure 1a shows the comparison between CML pts and healthy subjects. In CML pts, both PMN and MD-PBMC displayed shorter TRF-L, although the finding is more striking in PMN (mean telomeric loss in PMN 1932 pb; in MD-PBMC: 830 pb). Telomeric loss is more severe in young pts compared to older ones, resulting in loss of the association between TRF-L and age, typically seen in healthy subjects (Figure 1b). We found no correlation between TRF-L and previously mentioned clinical and demographic parameters. Telomere shortening was observed in both pts treated with or without TK inhibitors. When a multivariate analysis on pts and healthy controls was performed, the presence of CML resulted a stronger predictor of telomeric damage compared to age. *Conclusions*. Ph-negative hematopoietic cells repopulating the BM after successful treatment of CML display severe telomere erosion compared to healthy subjects. Indeed the previous CML history and treatment induced a proliferative stress comparable to that obtained in 38 years of physiological aging. The functional and genetic consequences of this premature aging are currently under investigation.

P055

ERYTHROID AND MEGAKARYOCYTE PRECURSOR SORTING FROM PH NEGATIVE MPD BONE MARROW ASPIRATES: PROPOSAL FOR A NEW USEFUL SAMPLING IN MOLECULAR DIAGNOSIS AND DISEASE MONITORING

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The diagnosis of Philadelphia negative myeloproliferative disorders (Ph^- MPD) has been recently improved by the identification of new molecular markers such as the JAK2V617F mutation or less frequently mutations in the JAK2-exone12 or in the TPO receptor (cMPLL/K515W). In our previous study we demonstrated how GATA1 was up-regulated in the bone marrow aspirates from patients affected by ET and PV comparing with the other MPD such as PMF or CML as well as in reactive secondary hyperproliferative conditions, speculating a possible role of GATA1 mRNA measurement as an additional molecular marker in ET or PV patients. According to the similar results in ET and PV we hypothesized that the disregulation in GATA1 expression could take place at a very early stage of the erythroid and MK maturation. For this reason we selected, by cell sorting, the $CD34^+$ CD38low precursor considered as the MK/erythroid progenitor (MEP), and then analyzed in those cells the GATA1 expression for ET and PV. We successfully collected a mean of 6288 cells in the MEP compartment of 7 ET, 4 PV patients and 3 controls starting from a median of 10×10^6 total bone marrow cells. We found that the GATA1 level was at least two log higher also in this very immature cell population isolated from MPD patients (median 2-DCt ET: 72, range 4-130; PV: 45, range 2-149) with respect to the normal subjects (median 2-DCt 6, range 0.8-8), suggesting this as a possible common pathway affected in ET and PV. With the above described method, because of the small number of samples, no definitive conclusions can be drawn but we could consider this as a valid option to define the clonal entity in a group of diseases whose features and behaviour is often variable and still lacking of a spin on treating or not. It could represent a way to discover the starting point of known or unknown molecular lesions associated with Ph negative MPD. Although valid guidelines are the most used tool to manage ET and PV patients, they do deserve parameters to quantify the disease and its progression. We propose the isolation of MEP as a possible target of clonality for detecting the active neoplastic clones and following the minimal residual disease.

P056

DETECTION OF A NEW AML1/ETO SPLICING VARIANT IN A PH^+ CML PATIENT

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The t(8;21) translocation corresponds to an in-frame fusion between the residues 1-177 of AML1 (21q22) and nearly the entire coding sequence of MTG8/ETO (8q22). The resulting chimeric protein inhibits in a dominant negative manner the transcriptional activity of wild-type AML1. The AML1/ETO fusion transcript is found in about 10-15% of de novo AML-2 and in a small portion of M0, M1 and M4; rarely, it has been reported as an additional abnormality associated to t(9;22) in chronic myeloid leukemia (CML) patients, both in chronic and blastic phase. We describe the clinical and molecular features of a new Ph^+ CML case rapidly evolving into blastic phase, in spite of Imatinib therapy, with an atypical AML1/ETO rearrangement. The chimeric transcript BCR/ABL, b2a2 subtype, was detected at diagnosis in a 51 year old male. The patient started with Imatinib therapy, nevertheless a progression to blastic crisis was observed after 4 months. Conventional cytogenetics revealed a t(9;22) in association to a t(8,21), in virtually all cells. Fluorescence in situ hybridization (FISH) analysis, using dual color dual fusion (D-FISH) probes for BRC/ABL and AML1/ETO confirmed this result. Reverse transcription polymerase chain reaction (RT-PCR) for AML1/ETO, followed by capillary electrophoresis showed a weak signal for the 396bp expected band and an additional unexpected product of 570bp. The possibility of an aspecific signal, was excluded by nested-PCR amplification with internal primers. Both amplicons differing 192bp were subjected to sequencing. The faster band of 260bp represented the classical AML1/ETO fusion transcript containing AML1 exon 1-5 and ETO exon 2-11, whereas the atypical large-sized band (452bp) derived from a new in-frame fusion including AML1 exon 6 retaining the complete AML1 Runt domain spanning residues 75-209. The AML1/ETO RT-PCR product is usually detected as a single band even if additional small amplicons of variable intensity can be seen. Three groups have identified alternative spliced out-of-frame variants consisting of additional ETO/MTG8 nucleotides, but in all cases AML1 breakpoint was at exon 5. The aberrant transcript observed suggests the presence of a new splice variant not previously described.

P057

CLONES SELECTION IN A CML PATIENT TREATED WITH TYROSYN KINASE INHIBITORS

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The increasing understanding of BCR/ABL functions and the pathways of cellular activation allowed to develop a rational targeted therapy for chronic myeloid leukemia (CML). Tyrosine kinase inhibitors (TKIs) actually give most CML patients the chance to achieve a complete hematological (CHR), cytogenetic and molecular response, with a good safety profile and minor side effects. However, since resistance to specific TKIs can occur at any time, an appropriate monitoring of therapy by cytogenetic and molecular analyses is a relevant tool to assess the therapeutic strategy. TKIs sensitivity may be affected by an inappropriate intracellular drug concentration or by mutations in the ABL kinase domain, that are reported to prevent or alter ABL-TKIs interaction. In addition, unknown BCR-ABL independent mechanisms of resistance might also be activated. In our study we present a CML case consecutively treated with imatinib, nilotinib and dasatinib. When treated with imatinib (12 months), the patient achieved a CHR in 3 months. FISH and RQ-PCR showed approximately 20% of $Ph1^+$ nuclei and BCR/ABL transcript respectively, whereas cytogenetic analysis showed 55% of Ph1+ metaphases. At the following controls, cytogenetic analysis rose to 100% (6 months) and 95% (9 months) $Ph1^+$ metaphases. No ABL mutations were detected. Therapy with nilotinib (6 months) was not effective and furtherly interrupted for toxicity (60-95% $Ph1^+$ metaphases). Screening for ABL mutations detected a deletion in exon 8 producing a frame-shift with consequent amino-acid change (from codon 475 to 483), and the creation of a stop codon at position 484. At the same time, the cytogenetic analysis showed the presence of 1 metaphase with i(17q). While the research for hemopoietic stem cell donor was activated, the patient underwent dasatinib therapy. At the cytogenetic control (at 2 months), the patient showed 100% $Ph1^+$, i(17q) metaphases, while the previously detected molecular alteration disappeared. In conclusion, the case reported showed an example of *in vivo* selection of genetically characterized tumor clones. In particular, the deletion detected on ABL was possibly a marker of the selected clone not contributing directly to drug resistance; while the i(17q) is a typical marker of tumor progression and probably cooperated for the loss of the proliferative control. In addition, this case suggests that if therapy is not able to strongly reduce blast cells and drastically stop proliferation, an expanding CML stem cell population could possibly accumulate fixing genetic alterations creating a large number of mutated sub clones which may be sequentially selected according to the proliferative/survival advantages or the drug sensitivity, laying the base for all TKIs unresponsiveness.

P058

ACTIVATING MUTATION VAL617PHE OF JANUS KINASE 2 GENE AND CXCR4 EXPRESSION IN PH1-NEGATIVE CHRONIC MYELOPROLIFERATIVE DISEASES

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Traditionally, Ph1-negative CMPD include Polycythemia Vera (PV), Essential Thrombocythemia (ET), and Myelofibrosis with Myeloid Metaplasia (MMM). The Val617Phe point mutation of Janus Kinase 2 gene (JAK2V617F) is believed to participate in the pathogenesis of Ph1-negative CMPD and occurs in the majority of patients with PV and approximately half of those with either ET or MMM. Disregulation of CXCR4 and disruption of the CXCR4/SDF-1 axis may play a role in the pathogenesis and disease progression of CMPD. In our institution we are following 25 patients with PV, 18 patients with ET and 15 patients with MMM. We used the allele specific polymerase chain technique for detection of Val617Phe mutation in all 58 patients with chronic myeloproliferative syndrome. Surface CXCR4 expression on circulating and bone marrow $CD34^+$ cells was measured flow cytometrically. We measured Val617Phe frequency as 80% (20/25) in PV, 50% (9/18) in ET, and 40% (6/15) in MMM. We found significantly elevated hemoglobin levels and platelet count together with very low serum level of erythropoietin in Val617Phe-positive polycythaemia vera and essential thrombocythaemia patient groups. However, white blood cell count and the frequencies of splenomegaly and other complications (thrombosis, bleeding, transformation to acute leukemia) were not significantly different between the mutation-positive and negative groups. The expression of CXCR4 on circulating $CD34^+$ cells was significantly reduced in patients with MMM as compared to normal controls and patients with PV and ET. By analysing immunophenotypic pattern of bone marrow $CD34^+$ cells we found in 10 out of 58 CMPD patients (i.e. 3 PV, 2 ET, 5 MMM) an over-expression of CXCR4 (as defined by CXCR4 mean fluorescence intensity ratio thresholds of more than 5). This subset of patients showed significantly higher levels of bone marrow blast cells and serum lactate dehydrogenase (LDH). No statistical association was found between JAK2V617F mutational status and the CXCR4 expression. The non-invasive mutation analysis of the Janus Kinase 2 Val617Phe is suitable for routine laboratory application and helps the differential diagnosis of chronic myeloproliferative syndrome. However current informations on disease-specific prognostic relevance of JAK2V617F are inconclusive, while our results warrant further investigation into the role of CXCR4 in CMPD and suggest that CXCR4 should be incorporated into the risk assessment of CMPD patients.

Chronic Myeloid Leukemia and Myeloproliferative Disorders (II)

P059

IMATINIB 600 MG IN ACCELERATED PHASE CHRONIC MYELOID LEUKEMIA INDUCES DURABLE CYTOGENETIC RESPONSES IN THE LONG-TERM: THE GIMEMA CML WORKING PARTY EXPERIENCE AFTER A 7-YEAR FOLLOW-UP

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Background. Imatinib mesylate (IM), a targeted inhibitor of the BCR-ABL tyrosine-kinase, is the standard of care for chronic myeloid leukemia (CML). In patients with CML in accelerated phase (AP-CML), the advent of IM significantly increased survival. However, few long-term data on the outcome of these patients based on large, prospective and controlled trials are available. *Aims.* The GIMEMA (Gruppo Italiano Malattie Ematologiche dell'Adulto) CML Working Party is conducting a phase II, multi-institutional prospective study (CML/003) to investigate the long-term effects of IM in AP-CML patients, treated with imatinib 600 mg daily. *Methods.* Patients were monitored for hematologic and cytogenetic response at 1-3 months intervals. A complete hematologic response (CHR) required the normalization of platelet and white cell differential count and absence of extramedullary involvement. The definition of return to chronic phase (RTC) required less than 15% blasts and less than 30% blasts plus promyelocytes in blood or bone marrow and less than 20% peripheral basophils. Cytogenetic analysis was performed with standard banding techniques. *Results.* One hundred and eleven patients were enrolled; median follow-up of the 41 living patients is 82 months (range, 73-87). In 107 patients (96%) was observed a RTC and 79 patients (71%) achieved also a CHR. Cumulative best rates of major cytogenetic response (MCgR) and complete cytogenetic response (CCgR) were 30% and 21%, respectively. CCgR was subsequently lost by 6 patients after 3 to 36 months from its first achievement (median, 10 months). A total of 90 patients (81%) discontinued IM, after a median time of 25 months (range, 1-86); only 7 patients (6%) discontinued IM because of adverse events. At last follow-up, 4 patients were alive in complete remission after allogeneic transplant, 16 patients have switched to second generation tyrosine kinase inhibitor and 21 patients were alive on IM therapy (13 in complete, 5 in partial, 1 in minor and 2 in null cytogenetic response). No late toxicities have been observed. The estimated rate of overall survival (OS) at 7 years was 43%, and was associated with the achievement of a MCgR (Figure 1). Conclusions. After a 7-year follow-up, IM continues to induce durable responses in patients with AP CML, with negligible toxicity.

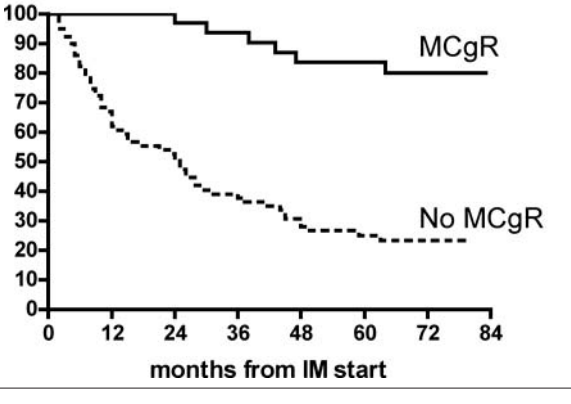

Figure 1.

P060

IDENTIFICATION OF C-ROS AS A NEW TYROSINE KINASE INVOLVED IN THE PATHOGENESIS OF CHRONIC MYELOMONOCYTIC LEUKEMIA

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The abnormal activation of tyrosine kinases are a common finding in chronic myeloproliferative disorders. Perturbation of RTK signalling by genetic alterations results in deregulated kinase activity and malignant transformation. c-Ros is an orphan RTK displaying transformation activity whose role has been described in case of neuronal neoplasia. Data coming from our previous gene expression study of TKs in CMPD identified Ros as overexpressed in different subtypes of CMPD. The aim of this study was to evaluate the involvement of Ros in the pathogenesis of chronic myelomonocytic leukemia (CMML) and to establish the effects of c-ros activation. Ros expression was evaluated by RQ-PCR in 92 samples collected from 54 CMML patients at diagnosis (42 PB and 50 BM) compared to healthy donors (30 PB and 30 BM). The protein amount and localization was analyzed by westen blot and immunofluorescence assay. In order to establish the effects of c-ros activation we generated a chimeric receptor containing the extracellular domain derived from epidermal growth factor receptor (EGFR) and the transmembrane and cytoplasmic domains from c-ros (ER). The chimeric receptor was then transfected in 293T cells. Transfected cells were then incubated with EGF ligand (100 nM) and proliferation and apoptosis evaluated by incorporation of 3H timidine and FACS respectively. We found that Ros is undetectable in healthy subjects but it is overexpressed in CMML (p<0,0001) in both BM and PB (median value of 2-Delta Delta Ct in BM is 280, range 10-63303 and 190 in PB, range 6-20900. WB confirmed the presence of c-Ros protein in CMML but not in normal controls. In addition, we found that ROS is highly expressed in $CD34^+$ cells, lymphocytes and monocytes from CMML patients but not in their respective counterparts. Sequence analyses revealed that no mutations are responsible for Ros activation. SNPs analysis exclude the presence of duplications or deletions. Moreover we found that the EGF induced activation of Ros affects proliferation but not apoptosis. This study demonstrates that c-Ros is abnormally expressed in patients with CMML The mechanism leading to that is still unknown, we could speculate that it is due to receptor overexpression. These new TK never described as involved in CMPD may be responsible, at least in part, for the proliferation defects which characterized CMML and could be a future target for molecular therapies.

P061

EXPRESSION OF MICRORNAS INVOLVED IN POST-TRASCRIPTIONAL REGULATION OF JAK-2 IN PHILADELPHIA NEGATIVE CHRONIC MYELOPROLIFERATIVE DISEASES

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MicroRNAs (miRNAs) are a large class of small non-coding RNAs that act as post-trascriptional inhibitors of gene expression through base pairing to partially complementary sites in the 3' untranslated regions of the targeted messenger RNA. miRNAs can play an essential role in the regulation of gene expression and thus dictating the cellular fate. Several authors have investigated the role of miRNA expression profile in patients with solid or hematological malignancies, showing that miRNAs are differently expressed in normal and tumor tissues. Therefore, it seems that miRNA expression profile could be a useful biomarker for diagnosis and prognosis of hematological disorders. We investigated whether miRNAs play any role in the pathogenesis of Philadelphia negative chronic myeloproliferative diseases such as Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Primary Myelofibrosis (MF). We performed a preliminary screening of miRNAs potentially inhibiting the translation of key genes involved in myeloid differentiation and maintenance of differentiated myeloid phenotype. We found that miR320 is a potential inhibitor of JAK2 and miR155 of SOCS1. We analyzed 12 bone marrow (BM) samples and 17 peripheral blood (PB) samples from PV patients, 27 BM samples and 23 PB samples from ET patients, 19 SP and 3 BM control samples. Preliminary results showed increased level of the immature form of miR320 (premiR320) in PV and ET patient; this enhanced expression could be due to a less efficient processing of the precursor into the active form. Indeed, 10/19 control samples showed higher level of the miR320 mature form in the BM and in the PB while a majority of PV patients and ET patients had higher level of the immature form (premiR320). Moreover, a majority of PV and ET patients with high levels of premiR, had the V167F JAK2 mutation. Thus a JAK2 mutated gene seems to be associated with higher premiR320 levels.

P062

INNOVATIVE PHASE I STUDY OF CONCOMITANT AND CONSECUTIVE TREATMENT WITH DASATINIB AND MK-0457 IN REFRACTORY PH+ CML AND ALL PATIENTS

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Background. MK-0457 is a pan-aurora kinase inhibitor with demonstrated activity against wild-type and mutated BCR-ABL, including the T315I form, as well as FLT3 and JAK-2. It is a promising molecule for the management of Ph^+ leukemias, in which the emergence of mutations in the ABL kinase domain still represents the main mechanism of resistance to TK inhibitors. In CML and ALL patients treated with a 5-day continuous infusion of MK-0457 at doses of 24-40 mg/m^2/hr every 14 days, an increased incidence of related adverse events was shown. Aim. In our Institution, an innovative Phase I clinical study of sequential and concomitant treatment with Dasatinib, previously administered for three months, and MK-0457 has been conducted. This combined activity suggests that MK-0457, in association with Dasatinib, would suppress the emergence of T315I and other resistant clone, improving upon the response rate for Dasatinib and the durability of response. The trial investigated two schedules of therapy: patients who achieved and maintained a major hematologic response after three months of therapy with Dasatinib (70 mg twice daily) received a 6-hour biweekly infusion of MK-0457 at 64 mg/m^2/hr, whereas patients who failed to achieve a major hematologic response received a 5-days continuous infusion of MK-0457 at 10 mg/m^2/hr, every 4 weeks. Biologically, the first schedule was demonstrated to suppress the emergence of Dasatinib-resistant clones, through a stronger inhibition of BCR-ABL, whereas the second one was showed to inhibit more potently Aurora Kinase activity. Results. Two patients with Ph^+ ALL and one patient with CML in myeloid blast crisis, previously unsuccessfully treated with imatinib, were enrolled in the protocol. The first two patients, both in hematologic response after three months of treatment with Dasatinib, subsequently received the 6-hour biweekly schedule, maintaining the haematological response. No haematological toxicity was described. The third patient, enrolled in progression disease, received the 5 days MK-0457 schedule of treatment. His peripheral blood count was consistent with a severe pancytopenia, which required frequent platelets and red blood cells transfusions. His bad clinical performance status was compromised by a severe hemorrhagic pleural effusion, responsible for moderate dyspnoea and severe asthenia. After one cycle of MK-0457, a complete recovery of the pulmonary disease and a complete hematologic response were obtained. *Conclusions.* The sequential and concomitant innovative administration of Dasatinib and MK-0457 represents a promising therapeutic strategy for refractory Ph^+ CML and ALL, showing a relevant haematological activity in a limited number of patients. Assessment of the benefit risk profile for this combination remains to be determined. *Supported by: European LeukemiaNet, AIL, AIRC, FIRB 2006, Fondazione del Monte di Bologna e Ravenna, Merck Sharp & Dohme.*

P063

AN INSERTION IN THE BCR-ABL KINASE DOMAIN CONTRIBUTES TO IMATINIB MESYLATE RESISTANCE

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The introduction of Imatinib Mesylate (IM) has produced major advances in the treatment of Chronic Myeloid Leukemia (CML) with >85% of patients (pts) achieving complete hematologic and cytogenetic responses after 5 years of treatment. However, 17% of chronic phase pts display primary resistance to IM or acquire secondary resistance to the drug and the incidence of resistance increases in the more advanced phases of the disease. Current studies indicate that multiple mechanisms contribute to IM failure including persistence of CML quiescent stem cells, BCR-ABL amplification and BCR-ABL kinase domain mutations (KDMs). The latter phenomenon accounts for 50–90% of IM resistance. BCR-ABL KDMs have been identified in more than 48 different residues and include single nucleotide substitutions that alter the conformation of the BCR-ABL KD or abrogate the physical interaction between the KD and IM. Recently, an intron-derived insertion/truncation mutation in the BCR-ABL KD has been proposed as an alternative mechanismin three CML pts undergoing tyrosine-kinase inhibitor therapy (Laudadio *et al*. J Mol Diagn, Feb 2008). Here we report the same insertion/truncation mutation in four CML pts displaying resistance to IM. The four pts (3 males and 1 female, median age 55 yrs) had a long history of disease (median time since diagnosis: 122 months) and underwent long-term IM therapy (median treatment duration: 56 months). All of them eventually failed IM and, at the time of drug resistance, three were on high dose IM therapy and one was receiving a second-generation tyrosine kinase inhibitor (dasatinib). At present they are all being treated with second generation tyrosine kinase inhibitors (two with nilotinib and two with dasatinib), with the two pts assuming nilotinib in complete hematologic and cytogenetic response. Unlike the report of Laudadio *et al*., in our pt cohort clonal sequencing revealed that the insertion was associated with both a wild-type and a mutated (M244V; F359V; H396R) BCR-ABL kinase domain. Preliminarystructural studies reveal that the insertion generates a truncated BCR-ABL protein that retains most of the KD but is devoid of the large unstructured region located downstream of the catalytic domain. Transient transfection experiments will determine the responsiveness of this truncated BCR-ABL to different tyrosine kinase inhibitors and its transforming potential in growth factor-dependent hematopoietic cell lines.

P064

IMATINIB INCREASES CYTOTOXICITY OF MELPHALAN AND DECREASES PROLONGATION OF G2-M PHASE IN HUMAN K562 CELLS

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The tyrosine kinase inhibitor imatinib represents the first line therapy for CML, but resistance phenomena can reduce the outcome of treatment. Combination of imatinib-mediated inhibition of BCR/ABL kinase activity with common anti-leukemic drugs may increase sensitivity of CML cells to genotoxic treatment. In our experiments we used K562 (a BCR/ABL-positive cell line) cells and tested the toxicity of a DNA-damaging anticancer agent, melphalan, in presence or absence of imatinib. Melphalan is a nitrogen mustard that acts by the alkylation of DNA. It exerts a cytotoxic effect through formation of monoadducts (that are repaired by nucleotide excision repair [NER]) and interstrand cross-links (that are repaired by a number of multistep pathways). BCR/ABL-positive cells seems to repair damage and activate DNA damage-dependent cell cycle checkpoints more rapidly than non-transformed cells because of increased activity of NER and other repair proteins. K562 cells were cultured alone (control) or with imatinib (IM) 1 µM for 24h. Mortality of IM treated cells was 45±5% respect to control. Cells were then resuspended and an equal number of cells were incubated for 1h with melphalan 20 µM or with culture medium. After drug treatment, cells were washed and resuspended in drug-free medium. The viability of cells was evaluated measuring luminescence by the ATP-lite1step assay (PerkinElmer) at 0, 24, 48 and 72 hs. Cells without any drug treatment exhibited an increased viability of 45±9% at 48-72 h. Cells not pre-treated with imatinib showed a mortality of 22±6% after 1h of melphalan (t0); 2±8% at 24 h; 8±6% at 48h and 26±9% at 72h. Cells pre-treated with imatinib showed a mortality of 68±8% after 1h of melphalan (t0) and the mortality was around 80±6% at 24, 48 and 72 hs. K562 treated with imatinib without melphalan started to grow after 48h showing a viability of 131%±8% at 72h. BCR/ABL expression increases genotoxic resistance also by prolongation of the G2-M checkpoint. In K562 not pre-treated with imatinib, cell cycle analysis exhibits accumulation in the G2-M phase at 24 h while cells pre-treated with imatinib showed a delay of the entry in this phase, that appeared at 48h only. In conclusion, inhibition of BCR/ABL activity by imatinib increased cytotoxicity of melphalan affecting the effectiveness of the DNA-repair pathways, and delayed accumulation in the G2-M phase and prolongation of this checkpoint for DNA repair.

P065

RATIO OF MUTANT JAK2-V617F TO WILD-TYPE JAK2 CORRELATE WITH MPD PHENOTYPES

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A single activating mutation in Jak2 gene was detected in 65-97% patients with polycythemia vera (PV), in 23%-57% with essential thrombocytemia (ET) and in 35%-57% with Primary Myelofibrosis (PMF). Recent studies have demonstrated the peripheral blood population contains clonal and polyclonal granulopoiesis; clonality correlated with the Jak2V617F allelic ratio. These data support the hypothesis that the ratio of mutant to wild-type Jak2 is critical for disease phenotype. To identify the Jak2 mutation in granulocyte-derived DNA and determine the allelic ratio we developed a microchip diagnostic platform (NMW NanoChip Molecular Biology Workstation, manufactured by Nanogen Inc, San Diego, CA) (Di Ianni *et al*., Leukemia 20:1895-1897, 2006). The system enables electronic deposition of biotinylated amplicons to selected pads. Using it we calculated the allelic ratio in 88 samples (33 PV, 45 ET, 10 PMF) that allelic specific PCR (AS-PCR) had shown to carry the Jak2-V617F mutation. The Nanogen system detected the Jak2-V617F mutation in all cases. The mutated allele was >40% in 14/33 (42.4%) cases of PV, in 9/45 ET (20%) and in 5/10 (50%) PMF. The quantification of the mutated gene with the electronic microchip gave a median of 35.5% (range 1.3-88.4) in PV; 12.2% (range 1,4-79) in ET and 40.4% (range 16.6-85.4) in PMF. Jak2 mutated allele was significantly higher in PV and PMF then in ET (ET *vs.* PV: $p< 0.002$, ET *vs.* PMF: $p<0.002$). Conclusions: The electronic microchip system detected the Jak2-V617F mutations in all cases. Significant higher level of mutated DNA were found in PV and PMF suggesting that different allelic ratios are indicative of the three different disease phenotypes we analysed.

P066

ROLE OF NEW GENERATION TYROSINE KINASE INHIBITORS ON OSTEOBLASTOGENESIS

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We and others have already demonstrated that Imatinib is able to induce osteoblastogenesis either *in vitro* and in chronic myelogenous leukaemia (CML) patients treated with this drug. Dasatinib (DA) and Nilotinib (NI), are new generation tyrosine kinase inhibitors presently approved for imatinib resistant CML patients. We therefore evaluated the effect of DA and NI on osteoblatic differentiation of Mesenchymal Stem Cells derived from bone marrow (BM-MSCs). BM-MSCs are multi-potent non-haematopoietic progenitor cells that differentiate into osteoblasts, adipocytes, chondrocytes, skeletal myocytes and nervous

cells. Mesenchymal stem cells (hBM-MSCs) were obtained from bone marrow samples of normal healthy adult bone marrow donors after informed consent, isolated by density gradient (mononuclear fraction) and cultured in standard medium (SC). Osteogenic differentiation of hBM-MSCs was induced in presence of 0.2 mM ascorbic acid, 0.1 µM dexamethasone and 10 mM -glycerophosphate (osteogenic medium, OM). Culture of normal human BM-MSCs were treated with SC or OM with and without DA 2 nM and or NI 100 nM. 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. DA 2nM and NI 100nM alone increased mRNA expression of RUNX2, OCN and BMP2 respect to SC: (DA; RUNX2 SC=1,59 0,20 *vs.* DA=2,09 0,16; OCN SC=2,57 0,28 *vs.* DA=3,2 0,14; BMP2 SC=1,55 0,19 *vs.* DA= 2,27 0,17); (NI; RUNX2 SC=1,59 0,20 *vs.* NI=4,2 0,31; OCN SC=2,57 0,28 *vs.* NI= 3,14 0,09; BMP2 SC=1,55 0,19 *vs.* NI= 4,16 0,27). BM-MSCs treated with OM and DA 2nM showed increased levels of osteogenic markers mRNA as compared to BM-MSCs cultured with OM only (RUNX2 OM=2,86 0,25 *vs.* OM+DA=4,41 0,41; OCN OM=3,59 0,17 *vs.* OM+DA= 3,6 0,28; BMP2 OM=2,84 0,28 *vs.* OM+DA= 4,43 0,30); NI 100nM too increased osteogenic markers (RUNX2 OM=2,86 0,25 *vs.* OM+NI=4,18 0,24; OCN OM=3,58 0,18 *vs.* OM+NI= 3,62 0,25; BMP2 OM=2,86 0,25 *vs.* OM+NI= 4,21 0,30). In summary, our data show that DA and NI increases osteogenic markers (BMP-2, Runx2 and OCN) mRNA expression in BM-MSCs, thus indicating that IM potentially favours osteoblastogenesis.

P067

EFFECT OF IMATINIB MESYLATE ON CARDIAC FUNCTION

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A few studies have appeared suggesting that Imatinib Mesylate (IM) may have cardiotoxic effects, which may lead to heart failure through decreased ejection function (EF), increased the left ventricular diameter and left ventricular disfunction. We investigated by echography a group of CML patients in long term treatment with IM, with no pause in their treatment and with no known previous cardiac problems. Thirty patients with median age 57 years, 17 male and 13 female, under treatment with IM for at least 4 years at standard dose entered the study. More than half patients (16/30) showed low/intermediate grade valvular regurgitation, which can be considered usual for the age. Only a single patient (3.3%) had serious cardiac problems: a mild left atrial dilatation, moderate tricuspidal regurgitation, related to right atrial dilation with mild pulmonary hypertension, pulmonary valvular regurgitation and posterolateral pericardial effusion in particular at level of the right chambers with a principle of diastolic collapse in lateral side of the right atrium, with normal left ventricle systolic function. This patient had been treated with the highest dose of IM (800 mg/die) for one year. We performed a case control study. Twentythree patients without preexiting cardiovascular problems, were pooled out from our series and they were compared with twentythree normal subjects with similar characteristics. Both groups were composed by 18 men and 5 female with the median age of 47.6±14.3. By standard doppler echocardiography, we compared structural and functional cardiac parameters between the two groups and we found some statistical differences. The patients treated with Imatinib showed increased interventricular septal thickness (p<0.03), left ventricular internal end-diastolic diameter (p<0.02), left ventricular internal end-systolic diameter (p<0.06), left ventricular mass (p<0.02), left ventricular mass index (p<0.01) and left atrial diameter (p<0.0001). These findings were unrelated to dose and length of treatment. Indeed, in a cohort of IM treated patients developing congestive heart failure (Kerkela *et al*., Nat Med) 7/10 patients had hypertension, 4/10 were diabetic and 4/10 had history of coronary artery disease. More serious adverse cardiac events, probably due to the pre-existing cardiovascular problems, which may be have remained undetected, and may be the basis for cardiotoxicity of IM. In conclusion, IM-related mild cardiotoxicity may be revealed by an accurate cardiac study. We suggest to perform an ecocardiographic study before beginning IM treatment, to be repeated during the follow-up. A larger case control study with long term follow-up may clarify the real impact of IM on heart failure.

P068

A BACKWARDS EVOLUTION IN A CASE OF MYELOPROLIFERATIVE DISEASE

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Thrombocytosis, erythrocytosis and marrow fibrosis are common and often overlapping features in myeloproliferative diseases (MPD). As typically seen in Chronic Myelod Leukemia (CML), and often observed for other MPD, disease progression leeds to fibrosis and blastic transformation. We report a case of a MPD with an uncommon sequence: Myelofibrosis first, than CML and finally Polycythemia Vera. In 1993 a 55 year old male was diagnosed to have Myelofibrosis. He had splenomegaly (cranio-caudal diameter 15 cm), WBC 7.000/ L, Hb 14,0 g/dL and platelets 380.000/ L; the blood film showed poichilocytosis with tear drop red cells; bone marrow (BM) biopsy showed increased reticulum. No metaphases were obtained for marrow cytogenetic analysis; a bcr/abl rearranged band (b2/a2 type) was found, coexistent with a germline configuration. The patient was in good health without any risk factor so a wait and see option was chosen. In June 2000 a typical picture of CML appeared: WBC 142.000/ L (43% neutrophils, 3% eosinophils, 8% basophils, 5% lymphocytes, 3% monocytes, 19% promyelocytes, 6% myelocytes, 13% metamyelocytes); Hb 13,1 g/dL and platelets 199.000/ L. Splenomegaly was increased. Bone marrow cytogenetic showed 100% Ph1 positive metaphases, and molecular analysis confirmed a clonal bcr/abl (b2/a2) positive myelopoiesis. He underwent hydroxiurea therapy then IFN therapy, because of intolerance to IFN since January 2005 the patient underwent Imatinib treatment. Starting July 2004, the hematocrit (Hct) increased gradually: on February 2006 Hct was 57.5%, requiring phlebotomies. Because of the unusual evolution of this MPD, we looked for the JAK-2 V617F mutation, which was found in all DNA samples banked, since diagnosis (1993). The most common sequence observed in MPD is erythrocytosis first, then thrombocytosis, then marrow fibrosis and finally possible blastic transformation. In this patient a rare case of going backwards was observed: (i) fibrotic BM lasting 7 years first; (ii) a bcr/abl positive clone expressing; (iii) erythroid type proliferation. Both bcr/abl hybrid gene and V617F JAK2 mutation were present. The coexistence of two molecular defects were not predictive of bad prognosis, since the disease course is lasting more than 15 years. This case may be a rare evidence of *in vivo* alternating proliferative activity of two clones, suggesting intriguing considerations on JAK-2 behaviour and role.

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P069

DYNAMICS OF BCR-ABL KINASE DOMAIN MUTATIONS IN PHILADELPHIA-POSITIVE LEUKEMIA PATIENTS AFTER SEQUENTIAL TREATMENT WITH MULTIPLE TYROSINE KINASE INHIBITORS

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Resistance to imatinib mesylate (IM) in chronic myeloid leukemia (CML) and Philadelphia-positive (Ph^+) acute lymphoblastic leukemia (ALL) is often caused by point mutations in the Abl kinase domain (KD) altering residues that are directly or indirectly critical for IM binding. Novel tyrosine kinase inhibitors (TKIs) have been rationally developed but each of them can be expected to retain its own Achilles heels. To assess a) which mutations develop *in vivo* under dasatinib or nilotinib treatment and b) how Abl KD sequences evolve under the selective pressure of sequential therapy with novel TKIs, we have monitored the mutation status of 95 IM-resistant pts before and during treatment with up to 2 consecutive novel TKIs (dasatinib, nilotinib). Forty-five pts (47%) had CML in chronic phase; 50 pts (53%) had CML in accelerated/blastic phase (AP/BP) or Ph^+ ALL. At the time of IM failure, 51/95 (54%) pts had KD mutations. After switching to a 2nd TKI (n=95 pts), 19/51 (38%) pts who had mutations at baseline as against 7/44 (16%; $p=0.02$) pts who did not have mutations at baseline subsequently relapsed with newly acquired mutations. Median time to relapse was 8 months (range, 1-22). In addition, 14/51 mutated pts did not respond to the 2nd TKI because of the mutation they were harbouring at baseline. After switching to a 3rd TKI (n=16 pts), 10/13 mutated pts as against 0/3 non-mutated pts relapsed with newly acquired mutations. Median time to relapse was 3 months (range, 1-5). Switch to a 4th TKI (MK-0457, PHA-739358) has so far been attempted in 3 mutated pts, but observation time is still too short. Newly acquired mutations in pts who failed dasatinib were T315I, F317L, V299L, T315A, F317I/S/V. Newly acquired mutations in pts who failed nilotinib were Y253H, E255V/K, L273M, F359V/I, T315I. We conclude that a) in IM-resistant pts treated with 2nd/3rd TKIs 90% of failures are associated with presence/emergence of mutations. However, the spectra of critical mutants are small and non-overlapping, the only exception being T315I; b) pts already harbouring mutations, especially those with CML in AP/BC or with Ph+ ALL have a higher likelihood of developing further mutations under the selective pressure of novel TKIs. It can be hypothesized that in these pts a higher genetic instability may foster rapid emergence of multiple mutations over time within the same or different Bcr-Abl-positive subclones, which are selected or de-selected depending on the specific TKI employed. Supported by AIL, AIRC, PRIN.

P070

MULTIDRUG RESISTANCE GENE HAPLOTYPE MAY BE A PREDICTOR OF UPFRONT RESISTANCE TO IMATINIB IN CHRONIC PHASE CHRONIC MYELOID LEUKEMIA PATIENTS

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The Bcr-Abl kinase inhibitor imatinib mesylate (IM) is the first-choice treatment in chronic myeloid leukemia (CML), but resistance has been observed in a proportion of patients (pts). Since blood and tissue concentrations of drugs are influenced by interindividual variations (single nucleotide polymorphisms, SNPs) in genes encoding drug metabolizing enzymes and drug transporters, we hypothesized that polymorphisms influencing the extent to which IM is actually delivered to target cells may account for the lack of response observed in some pts. IM is metabolised mainly by CYP3A4 and CYP3A5 enzymes, and, to a lesser extent by CYP1A1, CYP2D6 and CYP2C19 whereas IM transport in and outside cells is mediated by hOCT1 and MDR1, respectively. One hundred and twenty-one chronic-phase CML pts who achieved a complete cytogenetic response (CCyR) on IM 400 mg/d (responders) and sixty-three pts who have not achieved a CCyR (non-responders) on IM 400 mg/d were genotyped for the following SNPs: Cyp3A4*1B; Cyp3A5*2 and *3; Cyp1A1*m1, *m2, and *m4; Cyp2C19*2 and *3; Cyp2D6 *4; MDR1*6 and *8; hOCT1 Pro283Leu and Arg287Gly. Two hundred and seventy healthy individuals were analyzed in parallel to check for SNP frequency. MDR1*6 and *8 SNPs were under-represented in non-responders as compared to responders; however differences were not statistically significant. Analysis of MDR1 haplotypes revealed that responders had a marginally significant lower frequency of wild-type MDR1*6 and *8 as compared to non-responders (24.8% as against 33.3%, respectively; $p=0.045$). We did not find any significant differences in genotype frequencies between responders and non-responders (and between either groups and healthy individuals) as far as the other genes were concerned. Wild-type MDR*6 and *8 have both been shown to result in higher pump expression and activity with respect to the polymorphic counterparts and this may result in suboptimal intracellular concentrations of IM. Accordingly, MDR1 overexpression has been shown to be associated with IM resistance in CML cell lines. Analysis of a larger series of pts is now ongoing to further investigate the potential usefulness of MDR1 SNPs as predictors of resistance. In conclusion, our preliminary data suggest that genotyping for some MDR1 SNPs may find an application in identifying individuals at risk of resistance, for whom IM high-dose (800 mg/d) or alternative inhibitors might represent a more suitable therapeutic approach.

P071

AURORA KINASE B PROMOTES DEREGULATED PROLIFERATION AND RESISTANCE TO APOPTOTIC DEATH OF CHRONIC MYELOID LEUKEMIA PROGENITORS

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Aurora kinases (AK) are a family of conserved ser/thr kinases involved in cell cycle progression from G1 to S phase and chromosome segregation, the prelude to proper completion of mitosis. They contribute to the generation of binding sites for transcriptional repressor in the chromatin and, in particular, for heterochromatin protein 1 (HP1). Accordingly, their over-expression and/or constitutive activation may have a role in the pathogenesis and progression of human cancer. Here we report the impact of p210BCR-ABL tyrosine kinase (TK) on AKB enzymatic activity and interactions with the proteins of chromosome passenger complex (INCENP, Survivin and Borealin) involved in the recruitment of checkpoint proteins to kinetochores. The expression of BCR-ABL coding for either wild type (wt) or T315I mutated p210 protein in Ba/F3 cell line was associated with AKB hyper-phosphorylation. In both cell types, AK inhibitors significantly reduced AKB hyper-phosphorylation as well as p210BCR-ABL phosphorylation at Tyr245 in the SH2-linker domain. As expected, the two protein de-phosphorylation promoted cell recruitment in G1 phase of cell cycle, apoptotic death and induced a significant increment of polyploidy cells, the likely prelude to mitotic catastrophe. Moreover, we found a significant reduction of AKB expression in response to Imatinib mesylate (IM, 1 microM) in Ba/F3 cells expressing the wt p210BCR-ABL and to AK inhibitor in Ba/F3 cell expressing the wt and T315I mutated p210BCR-ABL isoforms. The effects of fusion protein on AKB transcription were mediated by histone H3 phosphorylation at Ser10 and acetylation at Lys14, critical for the recruitment of histone methyltransferase SUV39H1 at the gene promoter. AKB reduced expression and de-phosphorylation in response to *in vitro* exposure to AK inhibitors was confirmed in $CD34^+$ progenitors from 3 out of 4 Chronic Myeloid Leukemia (CML) IM-resistant patients expressing the T315I

mutation. We found a significant reduction of AKB transcript molecules in 9 IM-resistant CML or Ph1+ Acute Lymphoblastic Leukemias following *in vivo* AK inhibitor treatment, supporting the BCR-ABL impact on AKB transcription. In conclusion, our results are consistent with AKB participation in BCR-ABL-associated disease pathogenesis and progression to a drug-resistant phenotype, most likely proceeding from the induction of genomic instability. AK inhibitor dual effects on p210BCR-ABL TK and AKB might prevent the outcome of IM resistance and, therefore, improve CML prognosis.

P072

IMMUNOPHENOTYPIC CHARACTERIZATION OF NEOPLASTIC MAST CELL IN PATIENTS WITH MASTOCYTOSIS: COMPARISON BETWEEN FLOW CYTOMETRY AND BONE MARROW HISTOLOGY

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Mastocytosis is a group of diseases characterized by abnormal mast cell (MC) accumulation that may be limited to the skin (cutaneous mastocytosis - CM), or may involve other extra-cutaneous (EC) organs (systemic mastocytosis - SM). According to the WHO classification, diagnosis of SM requires that one major + one minor criterion, or three minor criteria together be fulfilled. The major criterion is the histological finding of multi-focal dense infiltrates of MC in BM or in another EC organ. The minor criteria are: abnormal morphology (spindle shape) of EC MC, serum tryptase levels >20 ng/mL, detection of D816V KIT mutation in BM, and expression of CD2 and/or CD25 on MCs. In the last ten years, flow cytometry (FC) proved to be a valuable tool to identify neoplastic MCs in BM samples of SM patients, thanks to its elevated sensitivity and specificity. The aim of our study was to compare the immunophenotypic characterization of neoplastic MCs by FC and BM histology in 67 adult patients with suspected SM. Each patient was evaluated as follows: basal serum tryptase level, BM aspirate and biopsy (stained for anti-tryptase, CD117, CD25, and CD2). Neoplastic MCs were identified by FC using a specific antibody combination (CD25/CD2/CD45/CD34/CD117). Additionally, we assessed the presence of D816V KIT mutation in BM by restriction fragment length polymorphism analysis. Based on the clinical, laboratory, immunophenotypic and molecular findings a definitive diagnosis was reached in 58/67 patients: 43 were diagnosed as SM (42 ISM, 1 MC leukemia), 11 as Monoclonal MCs Activation Syndrome, and 4 as CM. Using FC we could identify cells in the BM with SM features in 43/43 patients that were ultimately diagnosed as SM. By contrast, BM histology provided the major diagnostic criterion in 31/43 patients, and a minor criterion ($CD25^+$ MCs) in 11 cases. Moreover, FC detected abnormal MCs in 7/11 cases of MMAS; in this case, BM histology demonstrated $CD25^+$ MCs in just 3/11 cases. Overall, FC detected abnormal MCs in 51 cases. The median percentage of neoplastic cells was 0.1% on BM $CD45^+$ cells (range 0.004-40). Our data confirm that FC is a powerful tool to detect BM involvement by atypical MCs in all cases of SM, and can be considered as a valid alternative diagnostic approach in those patients in which invasive procedures, such as BM biopsy, are not applicable. Furthermore, FC may be a more sensitive technique than BM histology in the detection of hidden localization of abnormal MCs in MMAS.

P073

BRIT1 REGULATES G2/M CHECKPOINT IN CHRONIC MYELOID LEUKEMIA

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BRIT1 (BRCT-repeat inhibitor of hTERT expression), also known as microcephalin (MCPH1), acts as a regulator of both the intra-S and G2/M checkpoints. BRIT1 regulates mitotic entry by co-ordinating the regulation of Cdc25A and Cdk1-cyclin B1 activity both in ATR-Chk1 and ATR-independent signalling. In some solid tumors it has been demonstrated that BRIT1 level is inversely correlated with genomic instability. In our experiments we analyzed BRIT1 mRNA expression in K562 and in peripheral blood cells of chronic myeloid leukemia (CML) patients at diagnosis. Results were obtained using TaqMan Gene Expression (Applied Biosystem) by Real-time PCR and were normalized using glucose 6 phosphate dehydrogenase (G6PDH) as internal reference gene. The mean value calculated on 13 patients was 50% lower than 20 healthy donors (control). K562 showed BRIT1 mRNA levels 30 % lower than control. Because BRIT1 has a role in regulating mitotic entry, we assessed the capacity of K562 and CML cells to control G2/M checkpoint by cytokinesis block proliferation index (CBPI) assay. In particular, we used cells of two CML patients, one with BRIT1 mRNA level equal to controls (p1) and the other with BRIT1 mRNA level 50% lower than controls (p2). Following DNA damage, CBPI assay distinguishes cells that have not divided (because of proper induction of the G2/M checkpoint) from those that have undergone one (binucleated) or more nuclear divisions (multinucleated) and are thus more likely to develop chromosomal abnormalities. K562 and CML cells were pre-incubated for 2 h with 0,2 mM hydroxyurea (HU) or irradiated with 2,5 J/m^2 UV. Then, cells was pelleted, washed and incubated for 72 h with 5 µg/mL cytochalasin B, an inhibitor of cytokinesis. Cells were processed using standard light microscopy after Giemsa staining. Treated and untreated conditions were compared for the percentage of mononucleate cells respect to healthy donors cells. The percentage of mononucleate cells increased approximately of 15±4% in healthy controls and of 13±8% in p1 after treatment with HU or UV, indicating G2/M checkpoint arrest. On the contrary K562 and p2 showed this increase only after UV (49±9%) but not HU treatment, thus indicating that the G2/M checkpoint arrest system was activated after a strong DNA damage stimulus only. In conclusion, our study reported a defective G2/M arrest in CML cells with low BRIT1 mRNA levels. The identification of new biomarkers of disease progression may be important for novel therapeutic targets In this sense, studying BRIT1's role, mechanism of action and signaling may contribute to new therapeutical approaches in CML treatment.

P074

IN VITRO EFFECT OF SECOND MITOCHONDRIA-DERIVED ACTIVATOR OF CASPASES (SMAC) MIMIC COMPOUNDS ON MYELOID LEUKEMIA CELL LINES

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The disfunction of mechanisms regulating cellular apoptosis is a hallmark of cancer. Among processes leading to apoptosis, both caspase-dependent and -indipendent pathways have been reported. The X-Inhibitor of Apoptosis Protein (XIAP) binds caspase 9, 3 and 7, preventing their activation and, consequently, preventing cells from entering apoptosis. The Smac/DIABLO protein, released from mitochondria, binds XIAP as a dimer on the same caspase 9 (BIR3 domain) binding site. Similarly, the Smac protein interferes with the XIAP binding site for caspases 3 and 7, thus promoting both the extrinsic and intrinsic apoptotic paths. Several malignancies display overexpression of XIAP and a consequent caspase-dependent resistance to enter apoptosis. Therefore, XIAP inhibition via Smac mimic compounds represents a validated mechanism for intervention in cancer therapy. We tested 50 Smac mimic compounds (designed by CISI - Center for biomolecular Interdisciplinary Studies and Industrial applications of the Milan University) for their *in vitro* capacity to bind to the XIAP BIR3 domain and for their ability to inhibits the growth of the human leukemia HL60 and K562 cell lines (derived from patients with promyelocyitic leukemia and blastic phase-CML, respectively) as well as on normal $CD34^+$ hematopoietic progenitor cells. The effect of SMAC-mimic compounds on cell growth was evaluated by a colorimetric assay for the quantification of cell proliferation and viability based on the cleavage of the WST-8 tetrazolium salt by mitochondrial dehydrogenases. Following 72 hours-treatment with SMAC-mimic compounds, cells viability was evaluated measuring the absorbance by a microplate reader. The data were then expressed as

mean percentage of 3 replicates normalized to the untreated control. A strong correlation between the binding affinity to the XIAP BIR3 domain and the cytotoxic effect on the leukemic cell lines was observed. Interestingly the more promising compounds showed IC50 ranging from 0,3 to 1 microM on the HL60 cell line. With these compound, no cytotoxic effect was observed on normal controls at doses up to 80 microM. As Smac has been described to sensitize neoplastic cells for apoptosis, a combined treatment of Smac-mimics with conventional chemotherapeutic drugs is warranted, both on leukemic cell lines and on fresh leukemia samples, to investigate the possible synergistic effect which could permit to strength the effect even with lower doses of the Smac-mimic agents.

P075

EVALUATION OF TIE2-EXPRESSING MONOCYTES IN MYELOID DISORDERS

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Objectives. Several studies report on the pro-angiogenic contribution of bone marrow (BM) derived cells in hemato-oncologic diseases. In particular, endothelial progenitor cells (EPCs) and circulating endothelial cells (CECs) are established marker of vessel formation and damage. Recent studies present a new subset of BM-derived cells characterized by the expression of the angiopoietin receptor Tie2/Tek, the TIE2-expressing monocytes (TEMs). Circulating TEMs are a subgroup of tumor associated macrophage precursors that selectively promotes vessel formation *in vivo*. We here describe these parameters of angiogenesis in a group of different myeloid malignancies, diseases characterized by the neoplastic involvement of BM. *Patients and methods.* We analyzed 47 patients composed as follow: AML (10), ET (12), MDS (11), PMF (14). Additionally we examined 15 rheumatoid arthritis (RA) patients and 25 controls. CECs ($CD146^+$, $CD31^+$, $CD45^-$), EPCs ($CD34^+$, $CD133^+$, CD45-) and TEMs ($TIE2^+$, $CD14^+$) were evaluated by flow cytometry. *Results.* Hemato-oncologic population was characterized by elevated EPCs (p=0.02), normal CECs and TEMs values in comparison to controls. Regarding patient groups we found an increased number of EPCs in particular in PMF and AML groups (p=0.001) while CECs were higher in MDS (p=0.004). TEMs absolute number was similar to controls in all groups with the exception of PMF that showed markedly lower values (p=0.003). Interestingly, RA had a high TEMs (p=0.007). We also expressed TEMs number as percentage of mononucleated cells as reported before, in this case there were no differences between control and pathological groups. *Conclusion.* Patients with myeloid malignancies showed abnormal levels of serum EPCs and CECs. Conversely, TEMs number does not seem do change in these diseases with the notable exception of PMF, that is characterized by elevated CECs and EPCs but low TEMs. Interestingly, RA, a disease characterized by enhanced angiogenesis but normal macrophages, have high TEMs.

P076

MASKED MYELOPROLIFERATIVE DISORDERS AND JAK2 (V617F) DETECTION IN SPLANCHNIC VEIN THROMBOSIS: A SINGLE CENTER EXPERIENCE

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The diagnosis of Myeloproliferative Disorders (MPD) is often difficult in patients with splanchnic vein thrombosis (SVT), i.e. Budd-Chiari Syndrome (BCS) and non-cirrhotic Extra-Hepatic Portal Vein Obstruction (EHPVO), because of spleen enlargement and secondary pancytopenia that can mask erythrocytosis and thrombocytosis. The somatic mutation in the tyrosine kinase JAK2V671F has been described in MPD, specifically in the majority of patients with Polycythemia Vera (PV), in about half of cases with Essential Thrombocythemia (ET) and in one third of Primary Myelofibrosis (PMF). Recent diagnostic guidelines have included the analysis of JAK2V671F mutations in the diagnostic work-up of MPD. Since BCS and EHPVO have been usually described as idiopathic diseases, in this study we evaluated the prevalence and the levels of JAK2V671F mutation in a patient population affected by BCS and EHPVO. We analyzed 29 patients (median age: 38±9.8 years) with BCS (10), EHPVO (17), or both (2) followed at the Sapienza University of Rome. The JAK2V671F mutation was detected in 15 (51.7%) patients, while it was absent in the remaining 14. Among mutated cases, an heterozygous mutation (range: 2.7-52.9%) was detected in 8, while an homozygous status (55.7-90.4%) in 6. A diagnosis of PV was made in 3 patients affected by SVT with an heterozygous (2) or homozygous (1) JAK2V617F deletion. ET was diagnosed in 3 cases (2 heterozygous mutations and 1 homozygous mutation). In 5 patients with a diagnosis of PMF an homozygous or heterozygous deletion was found in 4 and in 1 patient, respectively. In the remaining 4 patients (26.6%) with JAK2 mutation, the bone marrow biopsy demonstrated the presence of initial marrow fibrosis, grade 0-1 and grade 1. In conclusion, the JAK2V671F mutation was detected in half of the cases with SVT and allowed to diagnose a MPD in a quarter of cases with masked disease. These results suggest that the investigation of the JAK2V671F mutation is warranted in patients with BCS and EHPVO because it can demonstrate the presence of a masked MPD, allowing a better clinical-prognostic disease definition and possible therapeutic intervention.

P077

JAK 2 GENE MUTATION AND WT1 OVER EXPRESSION IN PH1-NEGATIVE CHRONIC MYELOPROLIFERATIVE DISORDERS

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Ph1-negative chronic myeloproliferative disorders (MPD) represent a subcategory of hematological malignancies and are characterized by a stem cell-derived clonal proliferation of myeloid cells including erythrocytes, platelets, and leucocytes. Traditionally, Ph1-negative MPD include Polycythemia Vera (PV), Essential Thrombocythemia (ET), and Myelofibrosis with Myeloid Metaplasia (MMM). A high proportion (>50%) of patients with MPD carry a dominant gain-of-function V617F mutation in the JH2 kinase-like domain of JAK2 tyrosine kinase, conferring its constitutive activation and affecting downstream signaling pathways. The incidence of this mutation ranges from 65-97% in PV, from 41-57% in patients with ET, and from 23-95% in patients with MMM. For this reason, the V617F presence has been taking as a diagnostic value in MPD. WT1 is highly expressed in the bone marrow or peripheral blood of a variety of leukemias in comparison to normal bone marrow and normal progenitor cells. In Myelodysplastic Syndromes, increased WT1 expression is associated with higher blast counts and portends an early progression to AML; so, increased WT1 levels have a prognostic significance and are associated with a poor response to therapy. In our study we analyzed 58 chronic MPD patients and we found that 10 of them showed the presence of V1617F JAK2 mutation and the over expression of WT1 gene. These patients presented a greater splenomegaly, an increase of LDH levels, a boost of immature blasts in bone marrow and a partially reduced response to the therapy. The non-invasive mutation analysis of the Janus Kinase 2 Val617Phe is suitable for routine laboratory application and helps the differential diagnosis of MPD. Current informations on disease-specific prognostic relevance of JAK2V617F are inconclusive, while our results suggest further investigation into the role of WT1 over-expression in MPD and suggest that WT1 should be incorporated into the risk assessment of MPD patients.

P078

HYPOCHOLESTEROLEMIC EFFECT OF IMATINIB IN PATIENTS AFFECTED BY CHRONIC MYELOID LEUKEMIA

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Imatinib, a specific small molecule inhibitor of BCR-ABL, has become the standard drug therapy for CML, and has dramatically diminished the use of allogeneic stem cell transplantation. In our institution we are following 25 patients with CML (15 F and 10 M, median age: 45 years, r.: 19-66 years). All patients, at diagnosis, underwent a treatment with imatinib (400 mg/day). The therapy was well tolerated with minimal collaterals effects. After one year from the start of treatment 20 out of 25 patients achievied complete molecular remission of disease, while five patients with a sub optimal molecular response received a dose escalation of imatinib (600 mg and/or 800 mg/day). Actually all patients are alive: 22 out of 25 show a complete molecular remission, while three patients show a sub optimal molecular response. Interestingly, by analysing the routine biohumoral parameters we have found in 9 patients with initial high cholesterol levels (up to 300 mg/dL) a significative decrease of them with a normalization of serum cholesterol levels after six-eight months from the beginning of imatinib administration. All 9 patients did not use any hypocholesterolemic drug. Although there are several published data on the metabolic effects of imatinib administration little is still known about the effects of imatinib on lipid pathways, such as cholesterol metabolism. The cell signaling cascades provoked by Wnt proteins/beta-catenin play crucial roles to maintain homeostasis of a variety of tissues such as skin, blood, intestine, and brain, as well as to regulate proliferation, morphology, motility, and fate of cells during embryonic development. Several studies suggested that various tyrosine kinases associated with EGF-receptor and PDGF-receptor phosphorylate Tyr654 in beta-catenin. Imatinib mesylate (originally identified as an inhibitor of PDGF-receptor) inhibits tyrosine-phosphorylation of beta-catenin, suggesting that this chemical is also able to modulate the Wnt/beta-catenin signalling and consequently may reduce cholesterol de novo biosynthesis and increase uptake of exogenous cholesterol LDL-receptor mediated. In our hands this imatinib metabolic effect seems to be limited only to patients with previously high cholesterol levels. The significance of that and the clinical relevance of serum cholesterol level decrease in a long-term therapy, such as imatinib administration, are still unknown.

Chronic Myeloid Leukemia and Myeloproliferative Disorders (IV)

P079

INCIDENCE OF ABL KINASE DOMAIN MUTATIONS IN CHRONIC PHASE CHRONIC MYELOID LEUKEMIA PATIENTS WITH FAILURE OR SUBOPTIMAL RESPONSE TO FRONT-LINE IMATINIB TREATMENT – AN ANALYSIS BY THE GIMEMA WORKING PARTY ON CML

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Bcr-Abl kinase domain (KD) mutations are generally regarded as the main mechanism of resistance to imatinib mesylate (IM) in patients (pts) with chronic myeloid leukemia (CML). Nearly all studies, however, have focused on pts with advanced disease, where resistance is most often observed. Nowadays, the great majority of pts on IM are early chronic phase (ECP) pts receiving IM as front-line treatment. If, on one hand, the IRIS study demonstrated that response rates are high and relapse is infrequent in ECP, on the other hand we still know very little on the contribution of KD mutations to resistance in this subset of pts. Since January 2005, we have assessed the mutation status of 122 ECP pts on IM who were referred to our laboratory because their response was defined either as failure (n=77 pts) or as suboptimal (n=45 pts) according to European LeukemiaNet recommendations. Mutations were detected in 22/77 (29%) pts who failed IM: 1/3 pts who had no hematologic response (HR) at 3 months, 0/4 pts who had no cytogenetic response (CgR) at 6 months, 2/12 pts who had less than partial CgR (PCgR) at 12 months, 3/16 pts who had less than complete CgR (CCgR) at 18 months, 8/25 pts who lost CCgR, 8/17 pts who lost HR. Mutations were M244V (n=2), G250E (n=2), Y253H (n=4), E255K (n=1), T277A (n=1), E279K (n=1), T315I (n=1), M351T (n=3), E355G (n=1), F359V (n=3), H396R (n=3). In 7 pts who progressed to accelerated or blastic phase shortly after, five had mutations: G250E (n=1), Y253H (n=2 pts), E255K (n=1 pt) and T315I (n=1 pt). Mutations were detected in 7/45 (16%) pts who had a suboptimal response to IM: 2/12 pts who had less than PCgR at 6 months, in 4/17 pts who had less than CCgR at 12 months, in 0/6 pts who has less than major molecular response (MMolR) at 18 months, and in 1/10 pts who lost MMolR. Mutations were M244V, Y253H, F317L, M351T (n=2), F359V, F486S. High Sokal risk pts seemed to have a higher likelihood of developing mutations, although this need to be confirmed in a larger series of pts. We conclude that a) in ECP pts who receive IM as front-line treatment Abl KD mutations are not the major mechanism of drug-resistance, suggesting that early inhibition of Bcr-Abl reduces genetic instability; b) the incidence of T315I is very low in the setting of IM-resistant ECP pts; c) pts with high Sokal score might be at higher risk of mutation development. We also confirm that P-loop and T315I mutations often precede or are associated with disease progression. Supported by AIL, AIRC, PRIN.

P080

INCREASED BIOAVAILABILITY OF TRANSFORMING GROWTH FACTOR BETA1 IN PLASMA FROM PATIENTS WITH PRIMARY MYELOFIBROSIS

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TGFbeta1, a cytokine largely involved in the regulation of hematopoiesis and of deposition of extracellular matrix, is produced in a latent inactive form that becomes bioactive after proteolitic cleavage *in vivo* and by acidification *in vitro*. Previous studies in primary myelofibrosis (PMF) showed an increased TGFbeta1 mRNA expression in peripheral blood (PB) mononuclear cells and increased circulating levels of total (latent+bioactive) TGFbeta1. Here, we assess the levels of circulating bioactive TGFbeta1 in patients with PMF, polycythemia vera (PV)

or essential thrombocytemia (ET), and in healthy controls (CTRLs). Platelet-poor plasma samples were obtained from 40 PMF patients, 24 PV or ET patients, and 17 CTRLs. Bioactive TGFbeta1 was assessed by CCL64 mink cell line; bioactive TGFbeta1 levels are expressed as percentage of inhibition of CCL64 proliferation (in the presence of 1/1000 diluted plasma samples) induced by 1/50 diluted plasma samples. Total TGFbeta1 was assessed after plasma sample acidification. The percentage of bioactive TGFbeta1 in the plasma of PMF and PV/ET patients (43.3%±4.4SE and 31.3%±6.2SE, respectively) was higher (p<0.0001 and p<0.04, respectively) than that of plasma of CTRLs (6.4%±3.3SE). After plasma acidification the mean increase in the percentage of CCL64 cell growth inhibition was 24.3% in PMF, 38.9% in PV/ET and 69.1% in CTRLs, indicating that bioactive TGFbeta1 predominates in the PB of patients with PMF, PV and ET. Interestingly, BM plasma samples of 4 PMF patients showed higher (p=0.03) levels of bioactive TGFbeta1 than those of 4 disease CTRLs, while TGFbeta1 in PB was comparable to that of PMF patients. Finally, we found that bioactive TGFbeta1 levels in 10 PMF patients and 8 CTRL platelet lysates showed a comparable percentage of CCL64 growth inhibition. On the contrary, after platelet lysate acidification, the inhibition of CCL64 of PMF platelet lysates was higher (p<0.03) than that of CTRL lysates. Taken together, our data indicate that bioactive TGFbeta1 is more abundant in the PB of patients with PMF, PV and ET than in CTRLs. However, when BM plasma samples are evaluated, patients with PMF show bioactive TGFbeta1 levels higher than those of patients with PV or ET. Elevated percentage of bioactive TGFbeta1 in PB and BM plasma of PMF patients suggests a defective regulatory mechanism of this cytokine that does not involve TGFbeta1 platelet production.

P081

CHRONIC MYELOID LEUKEMIA IN BLAST CRISIS TREATED WITH IMATINIB 600 MG: OUTCOME OF THE PATIENTS ALIVE AFTER A 6-YEAR FOLLOW-UP

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Background. Management of Blast crisis Chronic Myeloid Leukemia (BC-CML) is the most challenging entity in the treatment of chronic myeloproliferative disorders. The introduction of Imatinib (IM) has opened a new option in the treatment of BC-CML. Early results have shown the superiority of IM compared to conventional chemotherapy; however, the long-term outcome of these patients remains to be clarified. *Aims.* The GIMEMA CML Working Party conducted a phase II, prospective study (CML/003) to investigate the long-term effects of IM 600 mg daily in BC-CML. Focus of this study is to assess IM long-term efficacy, response duration and survival, and to characterize prognostic factors associated with a favourable outcome. *Methods.* Patients were monitored for hematologic and cytogenetic response at 1-3 months intervals. A complete hematologic response (CHR) required the normalization of platelet and white cell differential count and absence of extramedullary involvement. A return to chronic phase (RTC) required less than 15% blasts and less than 30% blasts plus promyelocytes in blood or bone marrow and less than 20% peripheral basophils. Cytogenetic analysis was performed with standard banding techniques; response was rated as usual. *Results.* Ninety-two patients were enrolled. Forty-six patients (50%) had a sustained RTC, and 24 patients (26%) achieved a CHR. RTC was subsequently lost by 22 patients, for a median duration of the second CP of 11 months (range 1-67). Sixteen patients lost the CHR, for a median duration of the CHR of 6 months (range 1-43). Sixteen patients (17%) had a cytogenetic response (9 complete, 1 partial, and 6 minor or minimal). CCgR was subsequently lost by all but 2 patients after 2 to 12 months from its first achievement, for a median CCgR duration of 7 months. The Kaplan-Meier estimated median survival time was 7 months, and the survival rates were 53% at 6 months, 29% at 12 months and 11% at 36 months. For the 10 patients who achieved a MCgR, OS was significantly better (p=0.001) (Figure 1). After a median follow-up of 66 months, 7 (8%) patients are alive: 3 patients are on IM treatment (1 in CHR, 1 in partial CgR and 1 in CCgR). Three patients are in complete remission after allogeneic transplant. One patient is alive in BC, after failure of a second-generation TKI. Conclusion: IM as monotherapy was valuable and safe in the short-term, but relapse rate was high and the longer term clinical outcome was not significantly influenced.

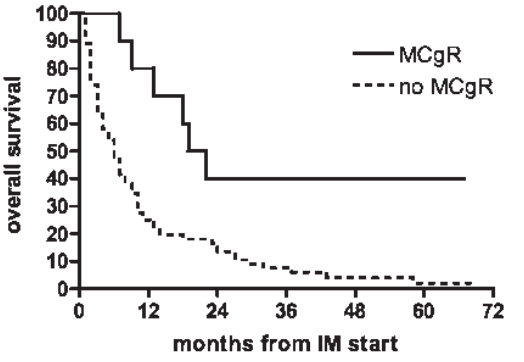

Figure 1.

P082

LONG-TERM MUTATION FOLLOW-UP OF PHILADELPHIA-CHROMOSOME POSITIVE LEUKEMIA PATIENTS TREATED WITH DASATINIB AFTER IMATINIB FAILURE: NEW BCR-ABL KINASE DOMAIN MUTATIONS ASSOCIATED WITH DASATINIB RESISTANCE ARE MAINLY DETECTED DURING THE FIRST YEAR / FOLLOW-UP

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Resistance to imatinib in Philadelphia-positive (Ph+) leukemia patients is often associated with selection of point mutations in Bcr-Abl kinase domain (KD). Dasatinib is a second-generation inhibitor with a different binding mode with respect to imatinib, that confers activity against many Bcr-Abl mutated forms. However, some mutations (T315I and, less frequently, F317L and V299L) retain resistance also to dasatinib and have been shown to be newly selected in patients (pts) who relapse. From February 2005 to January 2006, we enrolled in the international phase II trials of dasatinib a total of 45 pts with chronic myelogenous leukemia (CML) (n=35) or Ph$^+$ acute lymphoblastic leukemia (ALL) (n=10) who were resistant to or intolerant of imatinib. Clinical and mutation monitoring of these pts were regularly performed from pre-therapy up to the time of dasatinib discontinuation, due to intolerance, death for complications or primary or acquired resistance (n=24 pts). Median follow-up of all pts is 7 months (range, 1-38). Median follow-up of pts still on protocol (n=9 pts) is 36 months (range, 31-38). Relapses after an initial response have so far been observed in 16/45 pts. Fifteen out of 16 pts had advanced phase CML or Ph$^+$ ALL. In 13 out of 16 pts, relapse was observed during the first year of dasatinib therapy (median 6,5 months, range 4-12,5 months), while in 3 patients only it occurred during the second year (after 15, 18 and 33 months). In all cases, relapse was associated with newly detected Abl mutations (T315I, n= 8 pts; F317L, n= 5 pts; T315A, n=2 pts; F317I, n=1 pt; V299L, n=1 pt; 2 pts had multiple mutations). More detailed analysis will be presented. Our experience suggest that: a) resistance to dasatinib seems to be almost exclusively caused by KD mutations - suggesting that the higher potency with respect to imatinib can overcome Bcr-Abl gene amplification and that Src kinase inhibition may turn off Bcr-Abl-independent resistance mechanisms; b) novel dasatinib-specific mutant forms may be selected (F317V/I/S, V299L, T315A), but they may be sensitive to other inhibitors, including ima-

tinib; c) newly acquired mutations leading to relapse usually arise rapidly. The likelihood of mutation selection consistently decreases over time, and is mainly confined to advanced phase pts. Supported by AIL, AIRC, PRIN, FOndazione del Monte di Bologna e Ravenna.

P083

DASATINIB: OPTIMAL BRIDGE TO STEM CELL TRANSPLANT IN CHRONIC MYELOID LEUKEMIA BLAST CRISIS (CML-BC)

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Patients presenting CML-BC have a survival of 3-6 months and scarce response to imatinib. Dasatinib (BMS-354825) is an oral, multi-targeted kinase inhibitor, currently being used in pts with Imatinib-resistant advanced CML or relapsed/refractory Ph^+ ALL. Most of these pts will be evaluated for SCT, even though for them this curative therapy showed higher incidence of GVHD, VOD and TRM. We report here five pts affected from CML-LB who received Dasatinib prior to alloSCT. Donors were matched siblings (2), matched unrelated (2) or blood cord unit (1). Three were male and two female with median age 35,2 (18-48) years. First line therapies included Chemotherapy (VCR) plus high dose Imatinib. All pts after 2-5 months from diagnosis received Dasatinib 70 mg bid. T315I mutation occurred in 2 patients, Y253 and E255K in other 2 patients, and a non codified mutation in 1 patient. Dasatinib induced complete hematological response (CHR) in 3 pts, and complete (n=2) and partial cytogenetic response (PCyR) (n=1) prior to SCT. Two patients did not achieve CHR presenting 25% marrow blasts and 65% respectively prior to SCT. All pts were conditioned with myeloablative protocol. GVHD prophylaxis consisted of CSA and MTX (n=4) or micofenolate association until +30 (n=1). Pts received mobilized peripheral blood stem cell graft with 3,52-11,04×10^6 $CD34^+$ cells/kg (n=4) and cord blood unit with 0,1×10^6/kg $CD34^+$ cells (n=1). Dasatinib was stopped 6 days before transplant procedure. All pts successfully engrafted reaching ANC> 0.5×10^9/L on day +18 (11-33) and PLT >20×10^9/L on day +21 (11-50). Dasatinib was introduced again in 2 patients 30 days after SCT. One of them stopped therapy because of haematological toxicity after 2 weeks. All 5 pts presented chimerism 97-100%. Transplant related toxicities were grade I/II. None of the pts developed hyperbilirubinemia or VOD. Hyperacute extensive GVHD (Grade III) was observed in 1 patient on day +9. Four pts are alive, three in CMR, 1 in HR, 1 died of GVHD at 5.2 (2-13) months. We may conclude that in pts undergoing SCT following Dasatinib therapy there is no evidence of adverse effect on SCT outcome, organ toxicities, non-engraftment, GVHD or infections were never observed. Larger studies and longer follow-up are obviously indicated to confirm our preliminary results. Both T315I positive pts are alive in CHR. Dasatinib represents an efficient bridge to transplant to improve the outcome of this subset of patients.

P084

VARIANT PHILADELPHIA TRANSLOCATION IN EARLY CHRONIC PHASE OF CHRONIC MYELOID LEUKEMIA: RESULTS OF IMATINIB MESYLATE THERAPY (A GIMEMA WP ON CML ANALYSIS)

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Background. At diagnosis, variant Philadelphia (Ph) translocation occurring in patients (pts) with Chronic Myeloid Leukemia (CML) have been reported in 5-10% of pts. Some studies have suggested that variant Ph translocations may have an adverse prognosis with conventional chemotherapy or -interferon, while others have reported that have no impact on prognosis after Imatinib Mesylate (IM) treatment. Variant translocation could be three-way or four-way translocation (involving chromosomes 9, 22 and 1 or 2 additional chromosome respectively), associated or not with deletions of der(9) chromosome. AIM: To investigate the role of occurrence of variant Ph translocations on the response to IM in early chronic phase (CP) CML pts. *Methods.* A sub-analysis of 531 evaluable CML pts in early CP, have been performed within 3 simultaneously running trials of the GIMEMA WP on CML (CML/021; CML/022; CML/023). Median observation time was 30 months. Monitoring: hematologic, continuously; CC, FISH and molecular analysis were performed at baseline, 3, 6 and 12 months, and then every 6 months by local or reference labs. *Results.* At enrollment, 28 pts (5.3%) had variant Ph translocation: 1 showed variant Ph translocation by fluorescence in situ hybridization (FISH) but not by conventional cytogenetic (CC); 2 pts (7.1%) had a four-way translocation; 27 pts (96.2%) had a three-way translocation. In 5 pts (17.8%) translocation was associated with deletion of der(9). Only one carried an additional chromosome abnormality: t(7;19)(q21;p13). The two groups of pts, with or without variant translocation, were similar for age, Sokal risk and IM dose. At 12 months, 23 pts achieved complete cytogenetic response (CCgR; 82.2% *vs.* 83.9% in pts without variant), 2 pts reached partial cytogenetic response (PCgR; 7.1% *vs.* 6.6%), in 3 treatment was unsuccessful (10.7% *vs.* 9.5%). The 2 pts with four-way translocation reached CCgR, and 4 of 5 pts (80%) with deletion of der(9) reached CCgR. *Conclusions.* In the present large series of pts in early CP treated with IM therapy, we found no difference in cytogenetic response rates between pts with variant translocations and with classic ones. It will be discuss the eventual correlation between the complexity of mechanism of translocation genesis (one or two-step) and response to IM therapy. ACKNOWLEDGMENTS: University of Bologna (RFO), Fondazione del Monte di Bologna e Ravenna, European LeukemiaNet founds, MIUR PRIN 2005, Bologna AIL.

P085

TREATMENT OF PHILADELPHIA-POSITIVE CHRONIC MYELOID LEUKEMIA WITH IMATINIB: THE IMPORTANCE OF A STABLE MOLECULAR RESPONSE

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Background. The achievement of a major molecular response (MMolR) at 12 months is a surrogate marker of progression to accelerated/blast phase free survival (PFS) in chronic myeloid leukemia (CML) patients treated with imatinib (IM). *Aims.* We evaluated the prognostic value of the long-term prospective evolution of the MolR based on a retrospective analysis of 130 late chronic phase patients, who achieved a complete cytogenetic response (CCgR) with IM 400 mg. *Methods.* Two hundred and seventy-seven patients were treated with IM 400 mg daily after Interferon-alpha failure and 153 (53%) patients obtained a CCgR. For this analysis, 130 out of 153 patients (85%) were selected on the basis of the following criteria: 1) a CCgR confirmed at least twice; 2) at least 3 molecular tests performed after achieving the CCgR (median number of evaluable molecular tests: 5; range:3-8). Patients were monitored for cytogenetic and MolR every 6 months. Cytogenetic analysis was performed with conventional methods. MolR was assessed on peripheral blood by quantitative PCR (RQ-PCR, TaqMan) and results were expressed as a ratio of BCR-ABL:ABL %. MMolR was defined as a ratio BCR-ABL/ABL % less than 0.05; a complete MolR (CMolR) was defined as undetectable BCR-ABL transcript levels by RQ-PCR confirmed by nested PCR. *Results.*

In 71 patients (55%) MolR was always major (stable MMolR); in 19 (15%) MolR was occasionally less than major (unstable MMolR) and in 40 patients (30%) MMolR was never achieved (never MMolR). Patients with stable MMolR had longer CCgR duration and better PFS compared to patients with absent or unstable MMolR. The achievement of a MMolR, if maintained continuously, conferred a marked long-term stability of the CCgR (rate of CCgR loss: 4%): the probability of remaining in CCgR after 6 years was calculated by the Kaplan-Meyer method (Figure 1) and was 95% for patients in stable MMolR and 67% for patients with unstable or never MMolR (p<0.0001, log-rank test). Patients with stable MMolR have a significantly lower risk of losing the CCgR than patients with unstable (4% *vs.* 21%, p=0.03) and never MMolR (4% *vs.* 33%, p<0.0001). Finally, if a MMolR is not maintained continuously, the risk of losing the CCgR is higher but not significantly than if it is never achieved (33% *vs.* 21% p=0.5). Conclusion: These data confirm that achieving a MMolR is prognostically important but indicate that the prognostic value of achieving a MMolR is greater if the response is confirmed and stable.

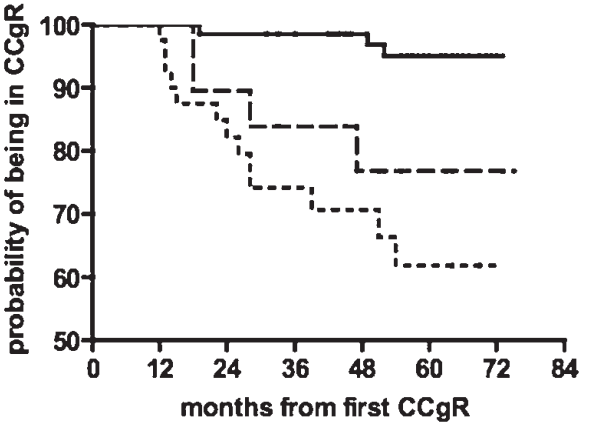

Figure 1.

P086

JAK2 V617F MUTATION IN ESSENTIAL THROMBOCYTEMIA: CORRELATION WITH CLINICAL CHARACTERISTICS, RESPONSE TO THERAPY AND OUTCOME IN A SERIES OF 275 PATIENTS

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Background. The JAK2 V617F mutation occurs in approximately half of the cases of Essential Thrombocytemia (ET) and is currently used in diagnosis. Aims. To determine the correlation between the JAK2 V617F mutation and baseline clinical characteristics in a cohort of 275 ET patients followed at a single Institution, and to evaluate the impact of JAK2 V617F mutational status on thrombo-haemorrhagic risk, response to therapy and incidence of disease evolution. Methods. The mutational state of JAK2 V617F was determined using the ASO-PCR essay, on RNA obtained from MONONUCLEAR CELLS isolated from peripheral blood samples. Response to therapy was defined as platelet count<600×10^9/L for at least 2/3 of the follow-up. *Results.* 275 ET patients were analyzed. JAK2 V617F mutation was detected in 175 patients (64%), of whom 173 (98.8%) were heterozygous (Table 1). When compared with wild-type (WT) ET patients, mutated patients were older at diagnosis (p=0.0015) and displayed a significantly higher haemoglobin (p<0.0001), hematocrite (p<0.0001), and a higher incidence of splenomegaly (p=0.01). On the other hand, platelet count was significantly lower in patients V617F positive (p<0.0001); no significant difference in leukocyte count was found. All patients were followed for a median time of 83 months (range, 3-374). One hundred and thirty-five out of 175 mutated patients (77%) and 80/100 unmutated patients (80%) received cytotoxic therapy for a median time of 77 months (range, 3-364) and 90 months (range, 3-374), respectively, and specifically: Hydroxyurea alone (123 patients), Busulfan alone (24 patients), Interferon alpha alone (13 patients) or more than one agent (55 patients), without differences between the two groups. V617F positive patients had a significantly better overall response to cytotoxic therapy, with 87% of the patients achieving a response (*vs.* 70% in WT patients, p=0.01). No significant association was noted between the V617F positive genotype and thrombosis, occurring either at and post diagnosis. Evolution in acute leukemia (AL)/myelofibrosis (MF)/polycytemia Vera (PV) occurred in 4, 5 and 1 patients, respectively, and was not significantly higher in patients V617F positive (p=0.09). *Conclusions.* JAK2 mutation assessment is valuable in identifying patients with clinical characteristics mimicking Polycytemia Vera. However, thrombotic risk and incidence of disease evolution were not significantly influenced by the mutation.

Table 1 Baseline clinical characteristic of the 275 ET patients, according to the JAK2 mutational status.

	JAK2^{V617F} pos	*JAK2^{V617F} neg*	*p*
No.	175 (64%)	100 (36%)	
Median age, years (range)	63 (16-88)	55 (19-80)	0.0015
Sex, no. (m/f)	74/101	41/59	0.8
Median leukocyte no, 10^9/L	9 (4-19.9)	8.3 (2.7–17.7)	0.13
Median platelet count, 106 pt/L	712 (457-1523)	825 (452-2045)	<0.0001
Median Hb, g/dL	14.7 (10.3-15.2)	13.6 (10.2-15.3)	<0.0001
Median Hmt, %	44.4 (30.2-52.1)	40.9 (31.7-51.1)	<0.0001
Splenomegaly, no. (%)	21/175 (12%)	3/100 (3%)	0.01
Epatomegaly, no (%)	8/175 (4.5%)	4/100 (4%)	1
Previous thrombosis, no (%)	31 (18%)	11 (11%)	0.16
Previous haemorragies, no (%)	1 (0.5%)	5 (5%)	0.02
Median follow-up, months (range)	73 (3-364)	83 (3-374)	0.12

P087

LONG-TERM FOLLOW-UP OF 386 CONSECUTIVE PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA: SAFETY OF CYTOREDUCTIVE THERAPY, INCIDENCE OF DISEASE PROGRESSION, AND SURVIVAL

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Background. Despite the recent identification of the Jak2 V617F mutation in patients with Essential Thrombocythemia (ET), which will probably allow a better management of these patients, cytotoxic agents like Hydroxyurea, Busulfan and Anagrelide are to date the most commonly used treatments. *Aims.* To report results of the long-term outcome of 386 consecutive ET patients, followed at single Institution for a median follow-up of 9.5 years (range, 3-28.5). We evaluated the efficacy and safety of cytoreductive therapy, including the leukemogenic risk in the long-term, and explored the potential correlation of baseline characteristics and outcome.

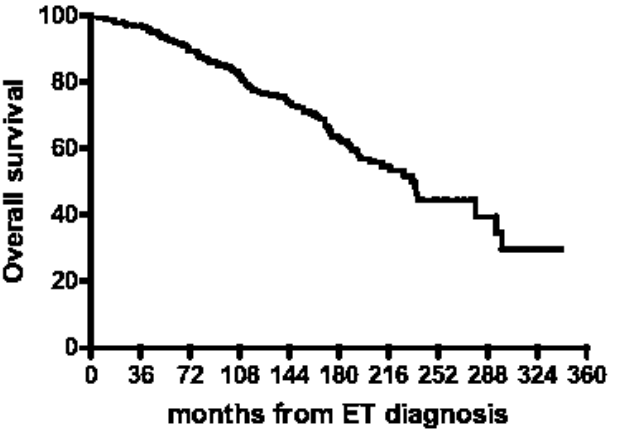

Figure 1.

Results. Cytoreductive therapy was administered to 338 patients (88%), obtaining a response in 86% of cases. Forty-five patients (12%) experienced a thrombosis. Among baseline characteristics, history of vascular events prior to ET diagnosis predicted higher incidence of thrombosis. Evolution in acute leukemia/myelofibrosis occurred in 8 (2%) and 19 (5%) patients, and was significantly higher in patients receiving sequential cytotoxic agents. Overall survival was 38% at 25 years (Figure 1) and was poorer for patients older than 60 years, with higher leukocytes count (>15×10^9/L), hypertension and mellitus diabetes at ET diagnosis. *Conclusions.* Cytoreductive therapy was effective in decreasing platelet number with negligible toxicity; however, thrombocytosis control did not reduce the incidence of thrombosis and, for patients who received sequential therapies, the probability of disease evolution was higher and OS was poorer.

P088

ITALIAN STANDARDIZATION OF QUANTITATIVE REAL-TIME RT-PCR FOR BCR-ABL ON BEHALF OF LABNET GIMEMA CML WP

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The RQ-PCR monitoring of BCR-ABL transcripts is integral part of the management of chronic myeloid leukaemia (CML) patients, but a common and widely diffused way for expressing results is an obvious prerequisite for its clinical use. During IRIS study, in order to harmonize results, three different labs performed the molecular analysis and defined an international scale (IS) for the BCR/ABL values. The standardized baseline (fixed at 100%) was defined as the median level of BCR/ABL in a set of 30 samples from untreated CML patients. Bias of the medians of the three labs respect to the standardized baseline were corrected by a conversion factor (CF). A value of 0,1% on this international scale, i.e. 3-log reduction respect to baseline, was defined major molecular remission and represents a surrogate marker for optimal response to treatment. We set-up a network of 20 labs distributed in Italy that use the IS to express the results of RQ-PCR of BCR/ABL. This initiative was coordinated, on behalf of GIMEMA – CML WP, by three labs, at Naples, Bologna and Turin Universities that are particular expert in this field and that have already aligned their results to the IS. In the first phase, in order to improve the reproducibility and comparability of results, all network centres implemented a same protocol, derived from EAC concerted action (Gabert, Leukemia 2003), covering all phases of the. In a subsequent step, standard RNAs containing 4 different levels of BCR/ABL transcripts were distributed among the centres. The lab specific CFs were calculated with the Bland&Altman test: the bias of participant lab to those of the coordinating centres, expressed on the IS, were calculated, outliers data removed, and the CF for each centre derived from the antilog of the bias. The conversion of participant lab to the international scale greatly improve the accuracy of the results. Indeed, the mean bias of participant labs passed from -0.22508 to 0.00013 (p<0.001). Finally, we validate CFs by a quality control performed distributing four patient samples with different levels of residual disease among participants. Also in this case, we verified a high level of alignment of *Results.* the CV of data distribution were 22%, 12%, 11% and 30% for the four levels of MRD tested (BCR/ABLIS of 12.5, 1.52, 0.15 and 0.02). Our data indicate that it is possible to set-up a network of labs specialized to MRD assessment in CML patients that are able to provide very accurate and reproducible results aligned to the IS.

Myelodysplasia

P089

PI-PLC-BETA1 PROMOTER GENE IS METHYLATED IN HIGH-RISK MYELODYSPLASTIC SYNDROMES AND ITS GENE EXPRESSION COULD PREDICT THE RESPONSIVENESS TO AZACITIDINE

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Aberrations in DNA methylation are frequently observed in various types of cancer, leading to inactivation and contributing to tumorigenesis. DNA hypermethylation can be reversed by demethylating treatments, such as azacitidine, which have become a promising therapeutic approach for patients with high-risk myelodysplastic syndromes (MDS). Nuclear lipid metabolism has widely been implicated in cell growth, differentiation, and neoplastic transformation. PI-PLCbeta1 is a key enzyme in nuclear signal transduction, and it is involved in many cellular processes, such as proliferation and differentiation. The presence of a monoallelic and cryptic deletion of the PI-PLCbeta1 gene, as well as an impaired regulation of the PI3K/Akt/mTOR axis, have been recently hypothesized to be implicated in mechanisms related to the evolution of high-risk MDS into Acute Myeloid Leukemia (AML), even though it is still unclear what are the molecular mechanisms underlying the progression of the disease. In the present study we showed that PI-PLCbeta1 promoter gene can be hyper-methylated in high-risk MDS patients. In particular, we used the Real-Time Methylation-Specific PCR (MSP) technique to quantify the degree of methylation in healthy donors as well as in patients affected by high-risk MDS before and during the treatment with azacitidine. Moreover, we employed a Real-Time TaqMan-based approach to demonstrate that the degree of the methylation could be related to the gene expression, in that the amount of PI-PLCbeta1 mRNA increases when the methylation decreases. Interestingly, only the expression of the PI-PLCbeta1b mRNA splicing variant, which is the nuclear isoform, seems to be directly affected by azacitidine, in that the patients we analyzed during the treatment showed changes in the expression of the PI-PLCbeta1b isoform only. Furthermore, the decrease of PI-PLCbeta1 promoter gene methylation and the subsequent increase of PI-PLCbeta1b gene expression seem to be related to a better responsiveness to azacitidine. Taken together, our findings demonstrate, for the first time, that the PI-PLCbeta1 promoter region is methylated in high-risk MDS patients and is correlated with the gene expression. Therefore, we feel that our data could pave the way for new therapeutic approaches in high-risk MDS patients, as the quantification of the expression of PI-PLCbeta1 could contribute to predict the responsiveness to azacitidine.

P090

POLYMORPHISMS OF DETOXIFICATION AND DNA REPAIR ENZYMES IN MYELODYSPLASTIC SYNDROMES

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Myelodysplastic syndromes (MDS) are multifactorial malignancies frequently associated with chromosomal aberrations and characterized by ineffective haematopoiesis. Exposition to DNA damaging agents may play an important role in the pathogenesis of MDS, particularly in therapy-related MDS. Potential DNA damaging factors include environmental xenobiotics, antiblastic drugs, ionizing radiations and endogenous factors. Detoxification and DNA repair enzymes protect DNA from damage, due to endogenous and exogenous sources, but when ineffective, the DNA damage can cause chromosomal instability leading to apoptosis or oncogenesis. Genetic background can modify the individual risk for genomic damage and for developing cancer. We performed a case-control study including 160 MDS (90 males, 70 females, median age 71 years), diagnosed according to the WHO classification as primary MDS (n=114), therapy-related MDS-AML (n=16), Myeloproliferative/Myelodysplastic Syndromes (n=26) and AML with multilineage dysplasia (n=4), and 160 matched controls. The impact of genomic polymor-

phisms (NQO1, CYP3A4, GSTA1, GSTM1, GSTT1, GSTP1, RAD51, XRCC3 and XPD) involved in xenobiotics detoxification and DNA repair on the risk to develop MDS was tested. Polymorphisms were detected using PCR-RFLP, multiplex PCR and mismatch PCR-RFLP techniques. Differences in genotypes distribution between patients and control subjects were evaluated using χ^2 test (Yates corrected). For expected cell values less than 5, the Fisher exact test and confidence intervals exact limits were preferred. The Kaplan-Meier method was used to estimate overall survival. A Cox model was used to identify independent prognostic variables. Similar frequencies of polymorphic variants NQO1, CYP3A4, GSTA1, GSTM1, GSTT1, RAD51, XRCC3 and XPD were observed in patients and control subjects. The GSTP1 105Val allele was overrepresented in MDS patients (62.1% versus 49.7%) and was associated to an increased risk of MDS (O.R. 1.66, C.I. 1.03-2.67; p=0.036). Moreover, MDS patients homozygous for GSTP1 105 Ile/Ile had a worse survival than patients with heterozygous Ile/Val or homozygous Val/Val phenotype (p=0.057, n=133 pts). In conclusion, although the GSTP1 Ile105Val polymorphisms is a risk factor for MDS, it is associated to favourable prognosis.

Table 1.

	Genotype	*Controls n (%)*	*n (%)*	*MDS OR (95% CI)*	*p-value*
	RAD 51-135	160	159		
	g/g	136 (85)	130 (81.8)	1.0 (Ref)	
	g/c	21 (13.1)	28 (17.6)	1.32 (0.79-2.23)	0.36
	c/c	3 (1.9)	1 (0.6)	0.35 (0.1-4.42)**	0.62*
	g/c & c/c	24 (15)	29 (18.2)	1.26 (0.67-2.38)	0.53
	XRCC3-241	159	152		
	Thr/Thr	48 (30.2)	48 (31.6)	1.0 (Ref)	
	Thr/Met	92 (57.9)	75 (49.3)	0.82 (0.48-1.39)	0.5
	Met/Met	19 (11.9)	29 (19.1)	1.53 (0.71-3.28)	0.31
T	hr/Met & Met/Met	111 (69.8)	104 (68.4)	0.94 (0.56-1.56)	0.89
	NQO1-187	159	152		
	Pro/Pro	106 (66.7)	97 (63.8)	1.0 (Ref)	
	Pro/Ser	47 (29.6)	48 (31.6)	1.12 (0.67-1.87)	0.75
	Ser/Ser	6 (3.8)	7 (4.6)	1.27 (0.35-4.76)**	0.89
	Pro/Ser & Ser/Ser	53 (33.3)	55 (36.2)	1.13 (0.69-1.86)	0.68
	GSTA1 Promoter	159	147		
	*A/*A	59 (37.1)	44 (29.9)	1.0 (Ref)	
	*A/*B	71 (44.7)	71 (48.3)	1.34 (0.78-2.31)	0.32
	*B/*B	29 (18.2)	32 (21.8)	1.48 (0.75-2.94)	0.29
	*A/*B & *B/*B	100 (62.9)	103 (70.1)	1.38 (0.83-2.29)	0.23
	CYP3A4 Promoter	160	154		
	a/a	153 (95.6)	142 (92.2)	1.0 (Ref)	
	a/g	7 (4.4)	11 (6.9)	1.69 (0.59-4.99)	0.41
	g/g	0 (0.0)	1 (0.6)	Undefined	0.48*
	a/g & g/g	7 (4.4)	12 (7.5)	1.85 (0.65-5.36)	0.38
	GSTP1-105	159	153		
	Ile/Ile	80 (50.3)	58 (37.9)	1.0 (Ref)	
	Ile/Val	61 (38.4)	73 (47.7)	1.65 (0.99-2.74)	0.053
	Val/Val	18 (11.3)	22 (14.4)	1.69 (0.78-3.64)	0.203
	Ile/Val & Val/Val	79 (49.7)	95 (62.1)	1.66 (1.03-2.67)	0.036
	XPD-751	154	150		
	Lys/Lys	60 (39.0)	61 (40.7)	1.0 (Ref)	
	Lys/Gln	74 (48.1)	70 (46.7)	0.93 (0.56-1.55)	0.86
	Gln/Gln	20 (13.0)	19 (12.7)	0.93 (0.43-2.04)	1
	Lys/Gln & Gln/Gln	94 (61.0)	89 (59.3)	0.93 (0.57-1.51)	0.85
	GSTT1	155	156		
	wt	127 (81.9)	118 (75.6)	1.0 (Ref)	
	del	28 (18.1)	38 (24.4)	1.46 (0.82-2.62)	0.22
	GST M1	155	157		
	wt	63 (40.6)	76 (48.4)	1.0 (Ref)	
	del	92 (59.4)	81 (51.6)	0.73 (0.45-1.17)	0.2

P091

HEMATOPOIETIC AND MESENCHYMAL PROGENITOR CELLS DEFICIT IN PEDIATRIC MYELODYSPLASTIC SYNDROME

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Myelodysplastic syndromes (MDS) are an heterogeneous group of clonal hematopoietic disorders characterized by ineffective hematopoiesis and increased risk of progression to overt leukemia. MDSs are more frequent in adults, particularly in elder patients. In children are a rare disease with differences in the biology at presentation, frequently associated with inherited or costituntional disorders. To date, the most of the studies aimed at investigate the biology of MDS and the interactions between hematopoietic stem cells and stromal cells, are referred to adult patients and only few data are available for children. In this study we investigated the proliferative potential of hematopoietic and mesenchymal progenitors in 26 MDS children and compared the ability of normal or myelodisplastic bone marrow stromal cells to support the hematopoiesis in long term cultures. According to the WHO classification, 21 patients were diagnosed as Refractory Cytopenia (RC) and 5 patients as Refractory Anemia with excess of blasts (RAEB). The clonogenic growth has been established at presentation in all cases and during the follow-up in 17 patients. A significant decrease of hematopoietic progenitors was detected in 13/26 patients and the *in vitro* deficit significantly correlated with the severity of cytopenia. The numbers of CFU-GM, BFU-E and CFU-GEMM were significantly lower in the 17 patients with cytopenia involving two or three lineages than in the 9 patients with a single cytopenia (Table 1). To establish the clonogenic potential of mesenchymal progenitor cells in MDS, the number of CFU-F has been evaluated in 17 patients and 33 normal bone marrow donors. The number of CFU-F was significantly lower in MDS than in normal bone marrow (1.6±1.4 *vs.* 7.3±5.6; p=0.0001). In 8/26 patients we set up primary long-term cultures from MDS bone marrow. The results show that in 4/8 cases the ability to form a confluent stroma *in vitro* was lacked and in 6/8 patients the stromal cells failed to provide an adequate support to hematopoiesis by inducing a lower recovery of CFU-GM in supernatant of long term culture compared to that obtained from normal controls. These preliminary results shows that in about half of the MDS children either hematopoietic or mesenchymal compartment were defective and stem cells defect correlated with the disease status. The improved knowledge of the biology of MDS may be helpful for a more appropriate clinical approach. Partially supported by GRANT. Ricerca Corrente n° 2006/02/R/001822. OpBG and Associazione Davide Ciavattini, onlus.

Table 1.

Cytopenia	*N° pts*	*CFU-GM*	*BFU-E*	*CFU-GEMM*
0-1	9	107.0±75.9	131.0±43.6	21.6±14.9
2-3	17	45.3±42.1	38.7±50.0	5.8±8.3
p		0.02	<0.0001	0.003

P092

5-AZACYTIDINE, VALPROIC ACID AND ALL-TRANS RETINOIC ACID IN INT-2/ HIGH RISK MYELODYSPLASTIC SYNDROMES: FIRST RESULTS OF THE GIMEMA MDS0205 MULTICENTER TRIAL

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Epigenetic changes have been shown to play a role and to cooperate with genetic alterations in the pathogenesis of myelodysplastic syndromes (MDS). The potential reversibility of DNA and chromatin modifications makes chromatin remodeling enzymes attractive targets for therapeutic intervention in this disease. We conducted a phase II study on the combination of the DNMT inhibitor 5-azacitidine (5-AZA), the histone deacetylase inhibitor valproic acid (VPA), and all-trans retinoic acid (ATRA) in patients with intermediate-2/high-risk myelodysplastic syndromes. Bone marrow morphology was centrally reviewed before enrolment. VPA was given at 600-1500 mg daily to reach a final plasma concentration above 50 microg/mL, then 5-AZA was added at a standard dose of 75 mg/sqm daily, subcutaneously, 7 days for 8 cycles. In case of minor response, stable disease or failure after 4 cycles, ATRA was added at 30 mg/sqm orally daily, on days 8-27 for 4 cycles. Treatment was continued in responding patients until response persisted. The protocol included 62 patients (43 males, 19 females, median age 67 years, range 53-83 yrs). Diagnosis was RAEB for 37 patients, RAEB-t for 21, and CMML for 4 patients. The IPSS was int-2 (1.5) for 46 patients and High (>2) for 16 patients. A valproic acid concentration between 45 and 55 microg/mL was reached in a median of 7 days (range 4-28 days). Three patients died before treatment started, while 58.9% of patients (95% C.I.: 50.1-69.1%) are alive at 12 months. Disease progression occurred in 15 patients. RBC transfusion needs significantly decreased from a median of 3 units (range 0-16) before treatment start to 0 (range 0-7) after 8 cycles. Neurological toxicity occurred in 6 patients. Our data show that the 5-AZA/VPA/ATRA combination is safe and feasible in poor prognosis MDS patients.

P093

TNF-ALFA EXPRESSION IN MYELODYSPLASTIC BONE MARROW BIOPSIES: IMMUNOHISTOCHEMICAL DETECTION AND SIGNIFICANCE

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Myelodysplasias (MDS) is a heterogeneous disease with ineffective hemopoiesis and variable risk of evolution into acute leukaemia. Low risk (LW) cases are characterized by increased apoptosis in marrow precursors, while in high risk (HR) ones apoptosis is decreased and proliferation increased. In early stages, several cytokines are involved in apoptosis, mainly TNFalfa and Interferon gamma, and TNF inhibitors have proven effective on peripheral cytopenia in LR cases. We analyzed the immunohistochemical expression of TNFalfa in the onset bone marrow biopsies (BMB) from 21 MDS patients, divided into 4 LR, 5 HR and 9 intermediate risk according to the IPSS criteria. Three patients (intermediate and LR) treated with ATG and ciclosporine, were included as immune-mediated MDS. The BMB were B5 fixed and paraffin embedded; the anti TNFalfa (R&D Systems) was incubated overnight at 1:25 dilution, after unmasking procedures (3 5'-cycles of microwave irradiation 900W in citrate buffer); the Envision method was used. Control BMB from reactive cases revealed cytoplasmic stain only in mature erythroid cells and occasional myeloid precursors. Similar stains were seen in MDS biopsies and scored as percentage of positive cells in the cellularity. Although the results are preliminary and referred only to 21 patients, the study aims to collect data in 55 cases, all provided with clinical, laboratory, cytogenetic data and onset BMB. TNFalfa expression turned out to be higher in LR MDS (average 35-40%), lower in HR MDS (average 20-25%) and variable in the intermediate risk group. Independently on the risk status, the immune-mediated cases always showed marked expansion of late stage erythroid precursors, high TNFalfa expression (average 40-50%) and the strongest staining intensity of the whole series. In conclusion, our results seem to confirm what reported in the literature regarding a higher TNF expression in LR MDS, and highlight a subgroup of immune-mediated MDS with a particularly strong expression of TNFalfa. A larger case series is mandatory to confirm the observed results in all subgroups, possibly find clearer cut-off values among different risk classes, understand the intense staining of all immune-mediated cases and possibly verify other parameters in the intermediate group to correlate with the TNFalfa results.

P094

EFFICACY OF A COMBINATION OF HUMAN RECOMBINANT ERYTHROPOIETIN + 13-CIS RETINOIC ACID AND DIHYDROXYLATED VITAMIN D3 TO IMPROVE MODERATE TO SEVERE ANAEMIA IN LOW/INTERMEDIATE RISK MYELODYSPLASTIC SYNDROMES

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The efficacy of human recombinant erythropoietin (rEPO) in myelodysplastic syndromes (MDS) has been generally detected in untransfused patients with "refractory anaemia" according to WHO. We treated 63 MDS patients, Refractory Anaemia with Excess of Blasts (RAEB) - 2 excluded, with 13-cis-retinoic acid (20 milligrams/day) plus dihydroxylated vitamin D3 (0,5 micrograms twice a day) more or less 6-thioguanine (40 milligrams/day), as previously described, in addition to intermediate- high dose rEPO (30-80.000 U weekly). Median age was 74; diagnosis was Refractory Anaemia in 16 patients, Refractory Anaemia with Ringed Sideroblasts (RARS) in 8, Refractory Cytopenia with Multilineage Dysplasia (RCMD) in 18, RCMD with Ringed Sideroblasts in 2, RAEB1 in 16, 5q- Syndrome in 2 and unclassified MDS in 1. IPSS score was intermediate 1 in 29 patients, intermediate 2 in 3, low in 12, undetermined in 19. All patients had Hb <9.5 g/dL and 70% of them required regular erythrocyte transfusions. Erythroid response, (according to new IWG criteria) was defined by a reduction in the transfusion requirement of at least 4 U in a 2 month period or by Hb increment > 1.5 g /dL in non- transfused patients. Treatment was well tolerated and erythroid response rate was 60%: 50% in RAEB1 and 64% in non- RAEB patients (n.s.). Overall response was not affected by previous transfusion requirement (63% vs. 58% in untransfused), IPSS and WPSS scores, endogenous EPO level and weekly rEPO dosage (30-50.000 U vs. 80.000 U). Median response duration was 16 months (2 - 59+). Median overall survival reached 14 months for RAEB1 and 55 months for non- RAEB patients, with a significant difference in the latter between responders and non responders (median 82 *vs.* 44 months; *p*:0.036). In conclusion, our combination therapy, independently from rEPO dosage, achieved in patients with unfavourable response predictors a rate of anaemia improvement comparable to the best obtained in lower risk patients by high-dose rEPO.

P095

PATIENTS WITH MYELODYSPLASTIC SYNDROMES DISPLAY SEVERAL T-CELL EXPANSIONS WHICH ARE MOSTLY POLYCLONAL IN THE $CD4^+$ SUBSET AND OLIGOCLONAL IN THE $CD8^+$ ONE

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Immune dysregulation seems to play a crucial role in the pathophysiology of myelodysplastic syndromes (MDS), suggesting the hypothesis that a transformation of normal stem cells can induce an autoimmune

T-cell response with the bone marrow as the target organ. The goal of this work was to further understand which was the degree of immune system perturbation, by analyzing the T-cell receptor (TCR) repertoire of distinct T-cell subsets. We specially focused on the determination of the complementarity determining region (CDR3) pattern of the lymphocyte expansions detected in the T-helper and cytotoxic subsets. The analysis was performed in 30 patients and 15 age-matched controls. We combined a CDR3 spectratyping analysis in separated helper and cytotoxic T-cells with a flow cytometric evaluation based on a panel of 24 beta variable (BV) family-specific antibodies costained with anti-CD4/anti-CD8. By using a qualitative approach, we first looked at the overall degree of TCR repertoire skewing in the two cell subsets, via spectratyping. The repertoire of MDS patients was mostly Gaussian in $CD4^+$ T-cells, whereas $CD8^+$ T-cells were characterized by an extremely high frequency of both skewed (mean 88 *vs.* 74%) and oligoclonal BVs (mean 28 vs. 10%) when compared to normal controls. We then determined by flow cytometry the frequency of quantitatively expanded T-cell subpopulations, showing in patients an increased number of oligoclonal lymphocyte subpopulations, in both $CD4^+$ (5 *vs.* 1%) and $CD8^+$ (5 *vs.* 2%) T-cells. Finally we compared the findings obtained by genotyping and flow cytometry, showing that among the BV expansions detected in $CD4^+$ cells in patients, only 4% had an oligoclonal profile, 33% were skewed and 63% Gaussian. On the other hand, as regards the BV expansions detected in $CD8^+$ cells, 34% showed an oligoclonal profile, while 58% were skewed and 8% Gaussian. The present study confirms that the immune system of patients with MDS is overall extremely contracted, especially in the $CD8^+$ T-cell subsets. Moreover, it is characterized by an extremely high frequency of selective proliferations of both cytotoxic and helper T-cells, which are however mostly polyclonal in $CD4^+$ cells and oligoclonal in the $CD8^+$ ones. The striking difference between the two cell subsets could be interpreted both in the context of a different HLA class restriction and, more specifically, considering the selective involvement of the cytotoxic subset in the anti-tumor immune surveillance.

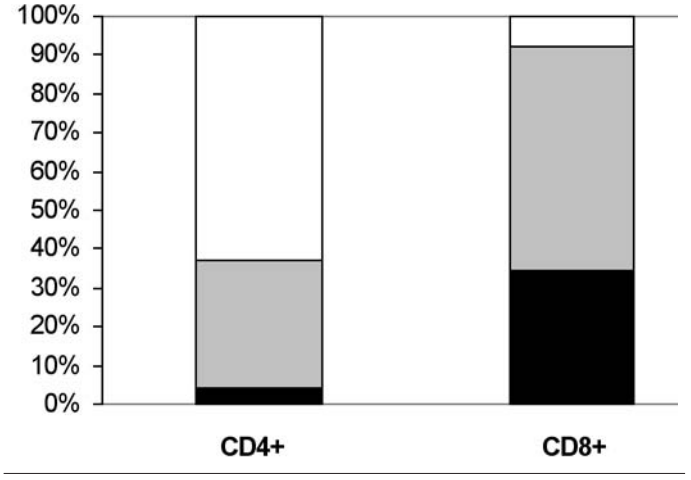

Figure 1. By comparing the findings obtained by genotyping and flow cytometry, we showed that of the 31 BV expansions detected in CD4+ cells in patients, only 1 had an oligoclonal profile (black), 9 were skewed (grey) and 17 Gaussian (white), corresponding to 4, 33 and 63% of total expansions, respectively. As regards CD8+ cells, of the 36 detected BV expansions, 9 showed an oligoclonal profile, while 15 were skewed and 2 Gaussian, corresponding to 35, 58 and 8%, respectively.

P096

WT1 AND CXCR4 EXPRESSION IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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WT1 is highly expressed in most acute leukemias, and its level of expression is associated with the presence, persistence, or reappearance of leukemic hematopoiesis. 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. We investigated WT1 gene expression and its association with the expression of the chemokine receptor CXCR4 on bone marrow $CD34^+$ cells of MDS patients. BM samples from 46 MDS patients (according to WHO classification: 20 RA, 10 RAEB I, 5 RAEB II, 4 RARS, 4 deletion of 5q, 3 MDS unclass) were tested for WT1 expression at diagnosis and every 6 months. WT1 gene expression was evaluated by methods of real-time quantitative PCR (RQ-PCR). Surface CXCR4 expression were measured flow cytometrically. At diagnosis, 29 BM samples (12 RA, 10 RAEB I, 5 RAEB II, 1 RARS, 1 MDS unclass) expressed WT1 transcript amounts greater than the ranges level. The degree of WT1 expression was highly correlated with the type of MDS, was much higher in RAEB I and II compared with RA, and other types, and increased during disease progression. Moreover, a significant correlation was found between WT1 expression levels, blast cell percentage and CXCR4 over-expression on blast cells (as defined by CXCR4 mean fluorescence intensity ratio thresholds of more than 5). The patients received only a supportive therapy if necessary. After 6 months, 11 patients (2 RA, 5 RAEB I, 4 RAEB II) converted to AML. All of these patients showed at diagnosis an high WT1 and CXCR4 expression and a further elevation of WT1 expression level after 6 months. Our data show that in most MDS, including a large percentage of RA and almost the total number of RAEB I and II, WT1 is expressed above the range observed in normal controls in BM and that its expression is directly correlated with the type of MDS. A strong association is present between the level of WT1 expression and the blast percentage and the CXCR4 over-expression. Our results justify further investigation into the role of CXCR4 in MDS and suggest that WT1 and CXCR4 should be incorporated into the risk assessment of MDS patients.

P097

IMMUNOSUPPRESSIVE TREATMENT WITH ANTITHYMOCYTE GLOBULIN AND CYCLOSPORINE IN AN ACCURATELY SELECTED SUBGROUP OF PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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Low-risk Myelodysplastic Syndromes (MDS) may share some of the features of acquired aplastic anemia (AA), and 30% of low-risk MDS patients improve their peripheral blood cytopenia following immunosuppressive therapy (IST) with antithymocyte globulin (ATG) and/or cyclosporine (CSA). Several clinical and laboratory features are associated with a higher probability of response to IST: bone marrow (BM) hypocellularity, the class II histocompatibility antigen DR15, low or intermediate-1 IPSS risk, younger age, shorter duration of red cell transfusion dependence (RCTD), normal karyotype, presence of Paroxysmal Nocturnal Hemoglobinuria (PNH) clones. As IST, especially if ATG is employed, may cause severe side effects in a population of prevalently old-aged patients, an accurate selection of patients for IST is required. On the basis of these data, in our Institution , from september 2006, 3 patients with transfusion-dependent low-or intermediate-1- risk MDS, showing bone marrow hypocellularity, age <70 yrs, and normal karyotype, were treated with ATG + CSA. Patient 1, a 25 yr-old female, came at our observation on 2/06, and had received only 2 PRC transfusions (duration of RCTD: 1 month). WHO diagnosis was of Refractory

Cytopenia with Multilinear Dysplasia (RCMD), IPSS was Int-1, bone marrow cellularity was 20%, karyotype was normal, DR15 was negative, and a small PNH clone was present. She started IST (ATG + CSA) on 9/06, and on day +36 she showed major erythroid Hematologic Improvement (HI): Hb 11.4 g/dL, without transfusion. Subsequently, from day +66, she developed a full picture of PNH, with progressive decrease of Hb, requiring again transfusion from 4/07. Now she is candidate for treatment with eculizumab. Patient 2, a 64 yr-old female, with RCMD; IPSS risk: Int-1, presence of DR15, and need of PRC and platelet transfusion (duration of RCTD: 1 month), was started on IST on 11/06, and showed erythroid and platelet HI from day +51 (Hb 9.9 g/dL, Plts 37.000/mmc); she is still maintaining HI after 18 months from ATG, still receiving CSA. Patient 3, a 66 yr-old female, coming from another Institution (previously treated with erythropoietin, without response), with RCMD; IPSS risk: Int-1, presence of DR15, and need of PRC and platelet transfusion (duration of RCTD: 17 months), started IST on 12/07. From day + 52 no more platelet transfusion were needed (platelet HI). Erythroid HI (HB 9.4 g/dl without transfusion) was observed from day +128.

P098

CD14$^+$CD56$^+$ MONOCYTOSIS, EXPANSION OF NK LYMPHOCYTES AND DEFECT OF HLA-E EXPRESSION IN MDS SYNDROME

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Myelodysplastic syndromes (MDS) are characterized by ineffective hematopoiesis with potential progression to acute myeloid leukemia (AML). Immune-mediated mechanisms have been suggested in the pathogenesis of MDS. The functional expression of CD94/NKG2 receptors has been correlated to Natural Killer (NK) activity regulation in physiological as well as pathological conditions. In this context, an altered expression of the non classical Human Leukocyte Antigen class I (HLA-I) molecule HLA-E,has been demonstrated to be relevant for NK activation. Here we describe an MDS patient showing peculiar CD14$^+$CD56$^+$ monocytosis, expansion of polyclonal CD56$^+$CD3$^-$ NK cells and defective HLA-E expression on monocytes and polymorphonuclear cells (PMN). Patient NK cells exerted *in vitro* autologous killing of PMN and expressed both the activating (CD94/NKG2C) and the inhibitory (CD94/NKG2A) HLA-E binding receptors. Here we propose that the polyclonal NK lymphocytosis has been critical for the pathogenesis of the expansion of myeloid progenitors lacking HLA-E expression. In addition, the occurrence of NK-dependent mechanism has been suggested to foster the myelodysplastic onset in the patient.

Acute Leukemias (I)

P099

DETECTION OF PROTEINASE 3 (PR3) GENE OVEREXPRESSION AND NUCLEAR DELOCALIZATION OF THE PROTEIN IN CORE BINDING FACTOR (CBF) ACUTE MYELOID LEUKEMIAS

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Proteinase 3 (PR3) gene codes for a serine protease with a broad spectrum of proteolytic activity. PR3 is involved in the control of proliferation of myeloid leukemia cells and it confers factor-independent growth to hematopoietic cells when abnormally expressed. The aim was to investigate the role of PR3 gene in leukemic haematopoiesis. We analyzed the expression levels of PR3 by RQ-PCR in 113 BM samples collected at diagnosis including 35 CBF AML. In addition 15 BM and 40 PB samples from healthy volunteers has been tested as control. PR3 protein amount and localization was analyzed by western blot (WB) and immunofluorescence. The transcriptional activity of CEBPA, which negatively regulates PR3, was investigated in parallel by RQ-PCR, WB and EMSA. Gain of function experiments were performed by transfecting HEK293T cell line with the full length PR3 sequence. We found that PR3 gene is significantly overexpressed in AML samples. The mean value of 2-deltadeltaCt is 740, (range 15-5043). Interestingly, patients affected by CBF leukemias showed significantly higher PR3 values compared to patients with normal karyotypes (NK):1571±1362 *vs.* 164±211 for M2 and 860±1177 *vs.* 160±110 for M4 (p<0,0002 and p<0,001 respectively) and lower CEBPA levels. EMSA assay demonstrated the absence of CEBPA DNA binding activity in CBF AML cells but not in NK AML. WB demonstrated the correlation between the mRNA and protein amount. Interestingly, immunofluorescence demonstrated the de-localization of the protein within the nucleus in CBF AML but it is cytoplasmatic in AML with NK. Transfection experiments in normal cells (HEK293T) with PR3 plasmid demonstrated that overexpression of PR3 did not significantly affect the proliferation rate and apoptosis. WB demonstrated that nuclear PR3 is able to cleavage the p65subunit of NF-kappaB into a p56 isoform which does not present transcriptional activity as confirmed by EMSA. In conclusion, PR3 gene expression and protein are significantly increased in AML, particularly in CBF leukemias in which the protein is abnormally delocalized within the nucleous. PR3 overepression is related to CEBPA downmodulation. The nuclear function of PR3 is probably implicated in the induction of a higher degree of chemosensitivity of the leukemic clone.

P100

MITOCHONDRIAL APOPTOSIS PROTEINS LEVEL PREDICT OUTCOME IN ACUTE MYELOID LEUKEMIA

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The unbalance between mitochondrial anti-apoptotic (bcl-2, bcl-xl, mcl-1) and pro-apoptotic proteins (bax, bad, bak) represents one of the key mechanisms explaining the high rate of treatment failure in AML. Moreover, the present availability of several pro-apoptotic molecules moved us to assess the impact of apoptotic pathways on AML prognosis. From 1995 to 2007, a large series of 420 non M3 AML pts, median age 63 years, treated with EORTC-GIMEMA protocols, were tested. The aims of our research were: 1) to correlate bax/bcl-2 ratio, as a measure of mitochondrial apoptosis, with other prognostic factors such as age and cytogenetics, and 2) to confirm that mitochondrial apoptosis is a pivotal and independent event. Bcl-2 and bax proteins were determined by multicolor flow cytometry. Bax/bcl-2 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. Two hundred-forty eight pts (59%) were bax/bcl-2 positive. Higher bax/bcl-2 and CD34 negativity (138/182; p<0.00001) or normal karyotype (101/139; p<0.00001) were closely asso-

ciated. No significant relationship was found between bax/bcl-2 and P-glycoprotein or FLT3-ITD. A higher complete remission (CR) rate was found in pts with higher bax/bcl-2 (68% *vs.* 32%, $p<0.00001$). Overall survival (OS) and disease-free survival (DFS) were shorter in pts with lower bax/bcl-2 (0% *vs.* 18% at 3.5 years; $p<0.00001$ and 0% *vs.* 19 at 2.7 years; $p=0.00007$). Also a shorter time to relapse was observed in pts with bax/bcl-2 <0.35 (3.3 months *vs.* 6 months, $p=0.011$). We investigated bax/bcl-2 within different age and cytogenetics subgroups to demonstrate its independent prognostic value. Lower CR rate and shorter OS were found in pts with lower bax/bcl-2 either within 251 pts older than 60 (26% *vs.* 64%, $p<0.00001$; 0 *vs.* 20% at 3.5 years, $p<0.00001$) or within 169 pts younger than 60 (57% *vs.* 84%, $p=0.0003$; 0 *vs.* 13% at 2.5 years, $p=0.001$). Lower bax/bcl-2 was associated with shorter OS and DFS within the normal karyotype (0% *vs.* 21% at 3.3 years, $p=0.0006$, Figure 1; 0 *vs.* 20%, $p=0.02$) and within the poor-risk cytogenetics subset (0% *vs.* 13% at 2.2 years, $p=0.003$; 0 *vs.* 17% at 1 year, $p=0.005$). The independent prognostic value of bax/bcl-2 was confirmed in multivariate analysis. We demonstrated the key prognostic role of mitochondrial apoptosis and therefore future therapeutic strategies should be focused on apoptosis-inducer drugs in order to improve outcome in AML.

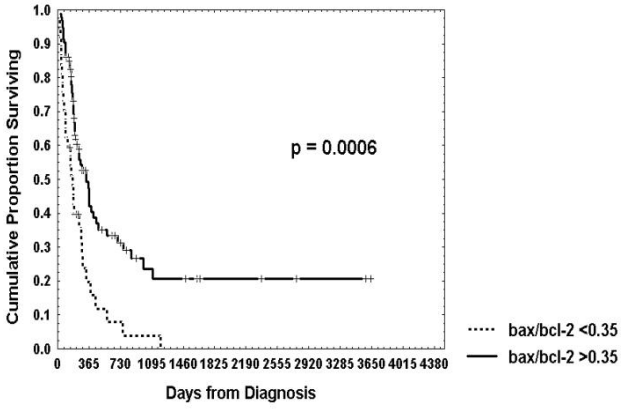

Figure 1. Overall survival by bax/bcl-2 within normal karyotype.

P101

IDENTIFICATION OF A "HOTSPOT" DNA REGION TARGETED BY TOPOISOMERASE II INHIBITORS IN AML1 GENE IN T-AML WITH T(16;21) TRANSLOCATION

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AML1 is a promiscuous gene known to be involved in multiple translocations in human leukemia and >31 chromosome translocations with 12 AML1 cloned partner genes have been identified so far. The translocation t(16;21) involving AML1 and resulting in the AML1-ETO2 fusion is a recurrent abnormality found in therapy-related acute myeloid leukemia (t-AML) associated with agents targeting topoisomerase II (topoII). We characterized at the genomic level the t(16;21) translocation in a 49 year old man who developed t-AML after treatment with mitoxantrone (MTZ) (15mg every 2 months for 1 year, cumulative dose 90mg) given for multiple sclerosis. At diagnosis, bone marrow showed a 35% infiltration by peroxidase positive granular blasts with some Auer bodies. Immunophenotype was CD33/HLA-DR/CD13/CD19/CD117^{+} and CD14/64/34/CD9^{-}. Screening for AML1-ETO1, PML-RARA, MHY11-CBF, and DEK-CAN fusion genes was negative. Mutational analysis showed no alterations in FLT3, cKIT and NPM1. The study of mutational status of the JAK2 gene showed the presence of the V617F mutation. Conventional karyotyping revealed a t(16;21)(q24;22) translocation which was confirmed by painting FISH analysis. Sequencing of the cDNA showed the presence of the AML1-ETO2 fusion transcript. Genomic long-range nested PCR followed by direct sequencing allowed us to precisely identify AML1 and ETO2 breakpoints at the DNA level in intron 5 and intron 3, respectively. AML1 intron 5 breakpoint was located at the nucleotide position 24785 (GenBank accession number AF015262). Interestingly, this region contained a ATGCCCCAG nucleotide sequence showing ~90% homology with a hotspot DNA region ATGCCCTAG contained in PML intron 6 gene previously identified in t-APL cases arising following treatment with mitox. By functional *in vitro* cleavage assays, this hotspot has been shown to correspond to be a preferential site of MTZ induced DNA cleavage by DNA topo II (Mistry *et al*, NEJM 2005). Microhomologies at the breakpoint junctions were indicative of DNA repair by the non-homologous end-joining pathway. This study indicates a wider distribution in the human genome, and particularly at genes involved in chromosome translocations observed in tAL, of DNA regions (hotspot) targeted by topoII inhibitors. As to the presence of JAK2 mutation in this case, our finding is in keeping with previous reports of t-AML with t(8;21) and JAK2 V617F suggesting that this may represent a cooperating alteration in AL pathogenesis.

P102

DETECTION OF DNA STRAND BREAKS AND CELL DEATH AS INDIVIDUAL PREDICTIVE RESPONSE TO GEMTUZUMAB OZOGAMICIN IN ACUTE MYELOID LEUKEMIA

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The lack of consensus treatment and the magnitude of clinical relapse are leading to the development of new drugs for the therapy of acute myeloid leukemia (AML). Gemtuzumab Ozogamicin (GO) is a FDA approved chemotherapeutic immunoconjugate which binds specifically to CD33. After internalization and dissociation, the calicheamicin derivative is translocated to the nucleus where induces DNA strand breaks, ultimately resulting in apoptosis. A variable clinical response to GO has been observed in spite of the presence of CD33 on blasts. Thus, in freshly isolated blasts from AML patients, the amount of GO-related DNA damage was evaluated by different methodologies to identify the possible mechanism of drug resistance and to correlate the *in vitro* individual response with the clinical outcome. For this purpose, patients enrolled in the EORTC-GIMEMA trial AML19 and patients with relapsed leukemia were included in the study. Ten ml of bone marrow were processed for cytofluorimetric evaluation of CD33. After blasts enrichment, the purity of samples was confirmed by FACS. Twelve patients fulfilled the inclusion criteria of the study. Cells were cultured in RPMI plus 10 percent FBS and exposed to 20 micrograms/mL of GO (Mylotarg, Wyeth). The saturation level of CD33 and p-glycoprotein (PGP) expression were measured by FACS. Apoptosis and DNA damage were evaluated at 3-6-12-24h after GO in treated and untreated blasts using the annexin/PI FACS technique, immunocytochemical evaluation of the expression of H2AX and comet assay. The exposure of blasts to GO resulted in a time dependent increase in cell death and DNA damage as measured by the three different methodologies. Genotoxicity increased up to 24h, at this time a strong induction of apoptosis was detected. Although PGP did not predict either clinical and *in vitro* response to GO, a correlation between *in vivo* drug resistance and low level of DNA damage was observed by both the comet assay and levels of H2AX expression. The comet assay was able to depict one group of patients showing DNA damage in all treated cells and a second group in which a significant portion of blasts did not showed DNA strand breaks. A long term follow up is ongoing to establish whether these cell behaviors may reflect different time to treatment failure. Thus, our study provides evidence on the feasibility of tailored therapy by the development of diagnostic tools for the evaluation of drug sensitivity in individuals affected by AML.

P103

EFFECTIVE *IN VIVO* INDUCTION OF NKG2D LIGANDS IN ACUTE MYELOID LEUKEMIAS BY ALL-TRANSRETINOIC ACID OR SODIUM VALPROATE

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Engagement of NKG2D by their ligands (NKG2DL), i.e. the human MHC class I-related molecules MICA and the UL16-binding proteins (ULBP)1-4, leads to the activation of cytotoxic lymphocytes and to the enhancement of anti-tumor effector functions. All these molecules are missing or expressed at very low levels on leukemic cells, although they can be up-regulated *in vitro* by all trans-retinoic acid (ATRA) or by the histone deacetylase inhibitor sodium valproate (VPA). Herein we show that MICA transcription was induced in leukemic cells 24h after administration of VPA to acute myeloid leukaemia (AML) patients, or after ATRA treatment in acute promyelocytic leukaemia (APL). ULBP2 transcription displayed a maximum increase at d3 for either treatment, while ULBP3 showed a slower and delayed kinetics following ATRA administration. Surface expression of NKG2DL is induced on leukemic cells by d3 of VPA or ATRA administration is detectable up to d7. After this time point, the percentage of blasts (<2%) was not sufficient to allow further evaluation of NKG2DL expression. Soluble (s) forms of MICA and ULBPs can be released by tumor cells and impair the function of cytotoxic lymphocytes; serum levels of sMICA and sULBPs have been proposed to be associated with disease progression in CLL and multiple myeloma. Thus, we measured sMICA, sULBP2 and sULBP3 in patients' serum before and during VPA or ATRA administration. Of note, serum levels of either NKG2DL did not vary upon VPA or ATRA treatment; only, sMICA was detectable in patients' sera and decreased after therapy. To verify whether the up-regulation of NKG2DL on leukemic blasts lead to effector cell activation, peripheral lymphocytes were isolated from patients after remission, stained with the anti-CD107a mAb, recognizing the lysosome associated protein LAMP1, and co-incubated with autologous leukemic cells obtained at diagnosis or after ATRA or VPA therapy. Of note, the percentage of $CD107a^+$ $CD8^+$ effectors significantly increased upon interaction with leukemic cells after ATRA or VPA therapy, indicating that they are triggered by autologous targets to degranulation. In conclusion, we provided evidence that up-regulation of NKG2DL on leukemic cells can occur *in vivo* upon administration of ATRA or VPA, with consequent activation of cytotoxic effectors. The therapeutic use of ATRA or VPA can be supported also because it may contribute to the enhancement of immune cell function., besides inducing leukemic cell maturation.

P104

USE OF PALLIATIVE PROGNOSTIC SCORE (PAP-SCORE) IN TERMINALLY ILL ELDERLY PATIENTS WITH ACUTE MYELOID LEUKAEMIA REFERRED TO A DOMICILIARY PROGRAM OF SUPPORTIVE AND PALLIATIVE CARE

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Home care has achieved a relevant role in the global management of patients with blood malignancies improving quality of life and reducing health care costs, especially in groups of unfit and elderly patients such as those affected by acute myeloid leukaemia (AML). In this clinical setting there is lack of consensus in predicting life expectancy and establishing the true terminal phase of disease. Unlike in haematologic neoplasms a palliative prognostic score (PaP-score) has been validated for terminally ill patients with solid tumors, based on predictive role of six factors (dyspnea, anorexia, Karnofsky index, clinical prediction of survival, white blood count, lymphocyte percentage). Here we report our attempt to apply the same prognostic score on a population of 36 terminally ill patients with AML, aged more than 65 years and undergone domiciliary assistance. In our division a haematologic home care service is active according to a protocol agreed in 1998 by university hospital, community health services and the fundraising organisation AIL (Italian Association against Leukaemia-Lymphoma-Myeloma). Eligibility criteria are: diagnosis of blood malignancy, age >18, low performance status, distance from hospital <15 km, availability of a care-giver, proper home logistics. In our study population, consisting of 36 patients (M=15, F=21) with a median age of 79 years (range 66-92), PaP-score was retrospectively calculated based on clinical information at the onset of terminal phase, that generally corresponded to the starting-day of home care. As shown in Table 1, patients have been divided in three risk-groups comparing the 30-day survival probability with actual duration of home assistance (91 days on average). Clinical estimation of survival was the most impacting feature on prognosis. Median PaP-score in patients deceased within the first month, was 11.1, matching the high-risk group, while in long-surviving patients PaP-score revealed a better survival probability. In the ongoing effort to improve the quality of remaining life in terminal elderly patients with AML a prognostic tool like PaP-score can represent a valid resource to predict life expectancy and to address decision-making processes toward a home care setting. The investigation of other prognostic factors, less subjective and more related to AML, such as peripheral blast percentage, bleeding, fever and transfusion requirement, might concur to define outcome and management in end-of-life care.

Table 1. Palliative prognostic score (PaP) – Maltoni et al., J Pain Symptom Management, 1999.

Criterion	*Assessment*	*Partial Score*
Dyspnea	No	0
	Yes	1
Anorexia	No	0
	Yes	1.5
Karnofsky Index	>30%	0
	10-20%	2.5
Clinical prediction of survival (weeks)	>12	0
	11-12	2
	7-10	2.5
	5-6	4.5
	3-4	6
	1-2	8.5
Total White Blood Count ($x10^9$/L)	<8.5	0
	8.6-11	0.5
	>11	1.5
Lymphocyte Percentage	20-40%	0
	12-19.9%	1
	<12%	2.5
Risk group	30-day survival probability	Total score
A (low-risk)	>70%	0-5.5
B (intermediate-risk)	30-70%	5.6-11
C (high-risk)	<30%	11.1-17.5

PaP-score in 36 elderly patients with aml referred to home care.

Target population	*Median duration of home care*	*Patients surviving > 30 days*
Group A (n=5, score=3.1)	153 days	4 (80%)
Group B (n=15, score=9.4)	125 days	12 (80%)
Group C (n=17, score=13.1)	44 days	9 (53%)
Overall (n=36, score=10.3)	91 days	25 (69%)
Target population	*Median PaP-score*	*Median duration of home care*
Pts died within 30 days (n=11)	11.1	20 days
Pts died from day +31 to 90 (n=13)	10.1	55 days
Pts died after day +91 (n=12)	8.4	138 days

P105

A DISTINCTIVE NUCLEAR MORPHOLOGY IN THREE CASES OF ACUTE MYELOID LEUKEMIA ASSOCIATED WITH LOSS OF HLA-DR EXPRESSION AND FLT3 INTERNAL TANDEM DUPLICATION

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In a 5-year survey of nonpromyelocytic/nonmonocytic acute myeloid leukemias (AMLs) diagnosed in our haematology department, we identified 3 cases containing distinctive, cup-like nuclear indentation in 10% or more of the blasts (definited AML-cuplike). In all cases, at least 10% of the blasts had a prominent, cup-like nuclear invagination spanning at least 25% of the nuclear diameter. In addition, a number of the blasts had a bilobed nuclear appearance in which a cleft could be seen spanning the entire diameter of the nucleus. These blasts demonstrated relatively scanty, pale basophilic cytoplasm with little cytoplasmic granularity, and only occasional Auer rods; also they showed near-uniform myeloperoxidase positivity, but did not show the intense, needle-like positivity characteristic of APL. By flow cytometry, all three cases demonstrated a predominant myeloid blast population with an abnormal immunophenotype: essentially uniform expression of CD123, loss of HLA-DR, partial expression CD34, CD117, CD56 and absence of CD133. Although lack of HLA-DR and CD34 expression is particularly associated with APL, and the invaginated nuclear morphology of AML-cuplike somewhat resembles the nuclear bilobation seen in many cases of microgranular APL, there was no evidence of the t(15;17) in these 3 cases that showed a normal karyotype by conventional cytogenetic. PCR analysis of the Flt3 gene was performed and all three demonstrated to harbor the internal tandem duplication (ITD) of Flt3 gene. To characterize AML-cuplike in an unselected series of AMLs, we analyzed 42 consecutive nonpromyelocytic/nonmonocytic AMLs diagnosed in our haematology department. Strikingly, in this unselected series, there was a statistically significant coincidence of invaginated nuclear morphology, loss of HLA-DR, and presence of the Flt3 ITD beyond that expected if these three features were unrelated, suggesting that AMLs with these three features may represent a distinct AML subset. However, because the clinical behavior of these AMLs is not known in detail, it is premature to conclude that these AMLs represent a distinct subtype deserving recognition under the WHO classification system and, for the present, are therefore classified as AML M1 or M2 under the FAB system. If these cases do turn out to represent a unique AML subtype, then additional features would appear to be uniform CD123 expression, low-to-negative CD34 and CD133, and a tendency to have a normal karyotype. Our detailed description of these features represents a necessary first step toward defining the clinical behavior and prognosis of AML-cuplike, including the potential therapeutic role for Flt3 inhibitors, although clinical follow-up of a larger series of cases will be required to confirm this.T

P106

SECOND COMPLETE REMISSION AFTER CNS RELAPSE OF APL WITH ARSENIC TRIOXIDE AND INTRATHECAL LIPOSOMAL CYTOSINE ARABINOSIDE. A CASE REPORT

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A 68 years old man in september 2005 was diagnosed with intermediate risk APL, kariotipic analysis t(15;17), PML/RARalfa rearrangment positive by RT-PCR. He received remission induction therapy with AIDA 2000 GIMEMA protocol. Molecular monitoring for PML/RARalfa was negative during consolidation therapy. In february 2007 he developed headache and vertigo. The initial laboratory evaluation revealed a normal leucocyte count of $4{,}3\times10^9$/L, hemoglobin level of 13,8 g/dl and platlets count of 131×10^9/L. Bone marrow examination showed normal hematopoiesis with no excess of blasts. Molecular analysis of bone marrow was positive for PML/RAR alfa rearrangement. A spinal tap was performed : the cerebrospinal fluid showed infiltration with $CD33^+$,$CD13^+$, DR^- blasts (680 cells/microliter). PML-ARalfa rearrangment was positive. On 23/2/07 he received the first intrathecal administration of 50 mg liposomal cytosine arabinoside. Fifteen days later treatment with ATO 0,15 mg/Kg/die was started and administered for 40 days. Bone marrow complete molecular remission was obtained after the therapy and the patient proceeded to maintenance with ATO for 5 weeks. Liposomal cytosine arabinoside was added to the treatment schedule every 21 days for a total of 4 intrathecal administrations. The CNS fluid showed a decrease of blast cells after the first administration (160 cells/μicroliter). A slight increase of cellularity was observed at the third lumbar tap (380 cells/microliter) , followed by a marked decrease at the fourth tap (20 cells/microliter) . The last two lumbar taps were negative at the molecular level. Post remissional therapy of CNS was 30 Gy irradiation delivered on the neuraxis. The patient is at present in continuous complete remission at one yaer from the stop of therapy, with persistently negative molecular monitoring of the bone marrow and no clinical evidence of central nervous sistem involvement.

P107

LONG-TERM DISEASE FREE SURVIVAL IN AN AML RELAPSED PATIENT TREATED WITH FLANG PLUS VALPROATE

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Hystone deacetylase (HDAC)-inhibitors represent a new class of anticancer drugs, with anti-proliferative and pro-apoptotic activities. Some of these inhibitors have also showed differentiation-inducing action in several carcinoma, leukemia and myelodisplastic syndrome cells. Antiepileptic drug valproic acid (VPA) has recently been discovered as a potent HDAC inhibitor. We report a 19 year-old man admitted at our division of Haematology in 2002, because of acute myeloid leukemia (M2). Cytogenetic analysis revealed trisomy 8 (intermediate cytogenetic risk status).Complete remission was achieved by induction ICE (Idarubicine, Ara-C, Etoposide) therapy. The patient had no HLA-matched sibling, and he was not considered a candidate for matched-unrelated allogeneic haematopoietic stem cell transplantation. Then a consolidation therapy with high doses of Ara-c was administered. The patient relapsed eighteen months later. FLANG (Fludarabine, Ara-C, Novantrone, G-CSF) re-induction treatment leaded to a second complete remission. At the same time, patient experienced epileptic seizures and therefore he started treatment with valproate. To date, after 46 months of follow-up, the patient is still in complete remission. The second remission is known to be usually shorter than the first one. We think valproic acid assumption modified our patient's disease evolution, by its activity on HDAC and by increasing cytotoxicity of chemotherapeutic drugs. *In vitro* studies on leukaemia cell lines demonstrated that enhanced cytotoxicity of valproate plus Ara-C is associated with valproate-induced up-regulation of cyclin D1 and Ara-C induced down-regulation of p27(e)kip1. It is remarkable that in our patient valproate effects on neoplastic cells have been obtained using standard anti-seizure dosing, without neurotoxicity and/or other adverse events, as also described by other investigators. In the literature several studies, showing beneficial activity of valproate in AML, myelodisplastic syndrome and other haematological diseases, are reported. Further studies are needed to evaluate efficacy and toxicity of VPA as a single agent and in combination with chemotherapy.

P108

SUCCESSFULL TREATMENT OF RETINOIC ACID SYNDROME WITH DEXAMETASONE IN ACUTE PROMYELOCITIC LEUKEMIA PATIENT

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The standard therapy for acute promyelocytic leukemia (APL) patients is based on all-trans-retinoic acid (ATRA). Retinoic Acid Syndrome (RAS) is a complication that has been noted to occur during the treatment of APL with ATRA. The diagnosis of RAS can be made based upon the presence of three of the following features: Fever, dyspnea, weight gain, hypotension, renal failure, pulmonary infiltrates, pleural effusion, and pericardial effusion; no single sign or symptom itself was considered diagnostic of the syndrome. Pulmonary manifestations, especially pul-

monary edema, are the most common presentation. Incidence of this adverse effect ranges from 6% to 27%. The pathogenesis of this complication is not completely understood. Several possible mediators have been identified: cathepsin G (a serine protease that enhances capillary permeability), cell adhesion molecules on APL cells such as CD15s(Lex) and integrins CD11a and CD11b, which interact with the endothelial cell receptor ICAM-1; and hematopoietic growth fctors such as IL-1beta, TNFalfa and IL-6. RAS can be potentially life threatening if not promptly recognized and treated. The onset of this syndrome is usually 5-21 days after ATRA treatment. We report a case of retinoic acid syndrome in a young male with APL (PML/RARalfa – bcr1) being treated with ATRA 45 mg/m^2/day per oral starting on day 1 and intravenous idarubicin 10 mg/m^2 on day 2, 4, 6 and 8. On day 19, he had oliguria with acute renal failure, high grade fever, hypotension, dyspnea and anemia (Table 1). We performed HRCT of the thorax that showed the diffuse alveolar damage: poorly defined centrilobular nodules, coalescent nodules with diffuse air space consolidation of both posterior lung zones with relatively sparing of the anterior lung fields and patchy zone of ground-glass opacity that suggesting the diagnosis of pulmonary haemorrhage and acute respiratory distress syndrome. The diagnosis of RAS was made and ATRA was withdrawn. Intravenous dexamethasone 10 mg every 12 hours was started. The patient's symptoms improved dramatically and bone marrow examination on day 48 was in complete remission. *Conclusions.* The use of all-trans retinoic acid to induce hematologic remission in patients with acute promyelocytic leukemia is associated in some patients with the development of RAS that is not uniformly accompanied by peripheral blood leukocytosis. Early recognition of the symptom complex of fever and dyspnea, combined with prompt corticosteroid treatment, may decrease morbidity and mortality associated with this syndrome.

Table 1.

Time	*day 18*	*0 (day 19)*	*+ 4*	*+ 8*	*+ 12*	*+ 24*	*+ 36*	*+ 48*
Creatinine mg/dL	1,4	2,8		3,2	2,0	2,0	1,8	1,7
SpO_2 %	98	82	88	92	98	98	98	98
HGB gr/dL	7,8	4,2		6,4	7,6	7,8	8,2	7,9
Fever °C	38,5	39,2	37,5	36,8	36,2	36,4	36,2	36
Blood pressure mm/hg	110/65	80/40	90/50	100/60	100/50	105/55	105/60	110/60
RBC transfutions		2		1		1		
WBC mm^3	450	1.170 Stop ATRA Start Dexa		860	760	830	890	715

Acute Leukemias (II)

P109

THE BCL-2 ANTAGONIST ABT-737 IS HIGHLY EFFECTIVE ON PRIMARY ACUTE LYMPHOBLASTIC LEUKEMIA CELLS

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The B-cell leukemia/lymphoma 2 (Bcl-2) family of proteins are important regulators of apoptosis and are frequently found aberrantly expressed, particularly in lymphoid malignancies. In this study, we investigated the cell cycle and apoptotic effects of ABT-737 (kindly provided by Abbott Laboratories), a Bcl-2 (BH3) inhibitor, on both lymphoid leukemia cell lines and primary acute lymphoid leukemia (ALL) cells. The lymphoid cell lines CEM and MOLT-4 were exposed to increasing concentrations of ABT-737 (from 100 to 1000 nM) up to 72 hours. A dose- and time-dependent cell growth arrest and induction of apoptosis was found in MOLT-4. In fact, measuring the subG0/1 peak at 48 hours, the levels of apoptosis increased from 8.5±4.5% (DMSO) to 13.6±1.4%, 27.1±2.6%, 44.1±5.9% and 78.5±1.2% at ABT-737 concentrations of 100, 250, 500 and 1000 nM, respectively (IC50=198nM). Conversely, CEM proved resistant (IC50=12.1microM). Western Blot analysis demonstrated that in the sensitive MOLT-4 cell line exposure to ABT-737 induces a dose-dependent down-regulation of Bcl-2, Bcl-xL and Mcl-1 protein expression, whereas in the resistant CEM cell line it did not. The effects of ABT-737 were then examined on primary blasts from 9 ALL patients. Primary ALL cells were cultured *in vitro* with ABT-737 (from 10 to 1000 nM) for 24 hours. A significant decrease in viability was observed at 10 nM (p=0.008) with a remarkable dose-dependent increase of apoptosis. In fact, Annexin V-positive cells increased from a mean baseline value of 16.8±8.8% to 43.6±22.8% (p=0.04), 66±21.3% (p=0.0001), 70.3±26.9% (p=0.04), 74.6±18.9% (p=0.03) and 76.2±11.8% (p<0.0001) in the presence of ABT-737 at 10, 100, 250, 500 and 1000 nM, respectively. A significant cell killing was demonstrated in 8/9 samples, including Ph-positive ALL cases. Following ABT-737 treatment, the detection of constitutive anti-apoptotic protein expression (Bcl-2, Bcl-xL and Mcl-1) showed in these samples a dose-dependent down-regulation which was not observed in the resistance sample. In summary, our study shows a potent growth-inhibitory and pro-apoptotic activity of the Bcl-2 antagonist ABT-737, at nanomolar concentrations, on primary cells from ALL samples regardless of their genetic abnormalities, suggesting a broad potential activity of the Bcl-2 family inhibitor in this disease.

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MICRORNA PROFILING IN ACUTE MYELOID LEUKEMIA

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Introduction. Naturally occurring microRNA (miRs) are an abundant class of 19-25 nucleotide non-coding RNAs that play important roles in cell proliferation and differentiation by acting as inhibitors of specific target genes at post-transcriptional level. Specific miR genes may contribute for oncogenesis working as classical tumor suppressor genes or as classical oncogenes, they may cause deregulation of target genes involved in cellular processes critical for leukemia development. MicroRNAs are important regulators of mammalian hematopoiesis (Fazi, 2005; Garzon, 2006; Debernardi, 2007) and their involvement in the pathogenesis of chronic lymphocytic leukemia has been demonstrated (Calin, 2002; Cimmino, 2005). Actually we have few data available on expression profile of miRs in acute leukemia (Isken,2007; Garzon,208; Marcucci,2008). To analyze the role of miRs in leukemogenesis we performed a quantitative expression study of set of 380 human miRNA genes in AML blast cells

and in normal bone marrow cells. *Methods.* Twenty-one cases of AML (1 FAB-M0, 4 M1, 4 M2, 3 M4, 4 M5, 1 M6 and 4 secondary leukemia) were enrolled in this study. In all cases immunological, cytogenetic and molecular studies were performed at diagnosis. Total RNA samples for the miRNA expression analysis were isolated from leukemic blast cells at diagnosis and from total bone marrow cells of four healthy subjects. To detect miRNA expression level, we used the specific assay available (TaqMan Low Density Array – Human MIRNA, 4342265 Applied Biosystems) PCR data were quantified using the SDS 2.3 software and normalized using the RNU48 as endogenous control. We analyzed gene expression by different statistical methods. the Significance Analysis of Microarrays (SAM), the Empirical Byes Analysis of Microarrays (EBAM). Results and discussion. Our preliminary data suggests a strong correlation of expression levels of some miRNA with morphological and genetic sub-type. In particular we found a up-regulation of miR-221, miR-222, miR-223 following the differentiation patterns (FAB M1 and M2 versus M4 and M5). Our data are in agreement with those reported by Fazi *et al* (2007) confirming the correlation of miR-223 expression levels to the stage of maturation block underlying myeloid leukaemia subtypes. We identifies some microRNA up-regulated, miR-222 miR-328 and miR-30c in NPM-1 mutated AML. According to Garzon *et al*. we found miR155 over-expression in FLT3-ITD mutated AML

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ARA-C, IDARUBICINE AND GEMTUZUMAB OZOGAMICIN AS SALVAGE TREATMENT IN ADVANCED ACUTE MYELOID LEUKEMIA PATIENTS

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Background. Long-term survival of relapsed/refractory acute myeloid leukemia (AML) patients remains a major problem, particularly in patients not eligible for transplant procedures. *Objectives*. To evaluate the feasibility and efficacy of adding Gemtuzumab Ozogamicin (GO) to salvage chemotherapy in relapsed/refractory AML patients. The main endpoints of the study were: 1) to assess the rate of second complete remissions (CR); 2) to increase the proportion of patients undergoing a transplant. *Methods.* The chemotherapy schedule was designed as follows: Ara-C 1 g/m^2 days 1-5, Idarubicine 8 mg/m^2 days 1, 3, 5 and GO 3 mg/m^2 on day 6 followed by Peg filgrastim 6 mg s.c. on day 8. All patients who achieved a CR were scheduled to receive an allogeneic stem cell transplant as soon as possible. A consolidation course was performed in case of a delay in donor availability. *Patients.* Twenty-four consecutive patients entered the study. The median age was 47 years (19-66); 54% of patients were males; 6 had refractory disease, 14 were in first relapse (median 1st CR duration: 9 months - range 3-48) and 4 in second or subsequent relapse. Four patients had high-risk cytogenetic features and 7 were FLT3 ITD positive; 9 patients had received a transplant procedure in 1st CR. *Results.* Eighteen of the 24 patients (75%) achieved a CR, while 6 proved refractory. No induction deaths were reported. Toxicity was mild; only 5 patients suffered grade III-IV mucositis and no case presented alopecia. The median time to PMN reconstitution above 0.5×10^3/μL was of 19 days (16-53) and 25 (14-54) for PLTS above 20×10^3/μL. Among the 18 patients who obtained a CR, 10 (55%) received a transplant procedure (1 autologous and 9 allogeneic), while 3 patients received DLI; two patients are waiting for a transplant and 3 have relapsed before the transplant. The 30 months projected probability of overall survival from the start of chemotherapy is 32%. Currently, 10 patients are alive in CCR with a median follow-up of 8 months (range 1-27). Conclusions: Salvage therapy using GO in combination with Ara-C and Idarubicine is feasible and well tolerated, providing a high CR rate and a useful bridge to transplantation. A longer follow-up and a prospective multicenter study are necessary to validate these results.

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AN ANTHRACYCLINE FREE REGIMEN BASED ON CONTINUOUS SEQUENTIAL INFUSION OF FLUDARABINE AND CYTARABINE FOR ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA

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The combination of fludarabine (F) with cytarabine (ARA-C) +/- G-CSF has been proven to be effective in poor risk patients with AML. In a phase II study, we investigated the efficacy and toxicity of a regimen including F + ARA-C administered as sequential continuous infusion (CI-FLA) in a series of untreated non-M3-AML patients aged more than 60 years. F at loading dose of 10 mg/sqm over 15 min at day 0, and after three hours and half ARA-C at a loading dose of 390 mg/sqm over 3 hours were given; at the end, F at 20 mg/sqm/ci/24 hours for a total of 72 hours and ARA-C at 1440 mg/sqm/ci/24 hours for a total of 96 hours were started. G-CSF was added at day +15 at a dose of 5 microg/kg. Patients achieving CR were programmed to receive an additional reduced course of CI-FLA, followed by G-CSF given at 10 microg/kg from day 15 in order to mobilize CD34$^+$ cells. Between June 2001 and November 2007, 112 patients received the treatment. Median age was 68 years (range 61-81). In 49 patients (44%) an antecedent myelodysplastic syndrome preceded overt AML. Cytogenetic analysis showed normal karyotype in 59 patients, complex karyotype or other unfavourable chromosomal abnormalities in 40 cases, no mitoses in 13 cases. Finally, 88 patients were affected by one or more concomitant diseases requiring specific treatment. Overall, 72 (64%) patients achieved CR, all but one following one course of CI-FLA. There were 21 induction deaths (19%), while 19 patients (17%) were refractory to induction treatment. The median number of days to neutrophil >0.5×10^9/L and platelet >20×10^9/L was 19 (7-34) and 19 (9-38), respectively. Documented infections occurred in 15 cases (13%). Fifty-nine patients out of 72 (82%) were eligible for the programmed consolidation course. Fifty-one patients were monitorized for the mobilization of CD34$^+$ cells, collection being successful in 37 (73%). Median number of CD34$^+$ cells/kg collected was 6.8×10^6/L (2-60.3), median number of apheresis being 2 (1-2). Thirty patients (27% of the overall population) received autologous stem cell transplantation (ASCT). Disease free survival and overall survival are 9 and 9 months, respectively. Survival at 5 years is projected to 20%. We conclude that CI-FLA is an effective and well-tolerated regimen for elderly patients with AML. Therapeutic results are encouraging as to CR achievement and ASCT feasibility and compare favorably with conventional anthracycline/ARA-C based therapy.

P113

MOLECULAR CHARACTERIZATION OF TAF1-NUP214 FUSION TRASCRIPT IN A SUBSET OF ADUL T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. NUP214 gene mapping at chromosome 9q34 encodes a FG-nucleoporin involved in nucleocytoplasmic transport. Template Activating Factor-I (TAF_I) transcribes two isoforms, TAF_I alpha and TAF-I beta bearing different N-terminal regions. TAF_I-NUP214 fusion gene was described in 1 case of acute undifferentiated leukaemia, 1 of acute myeloid leukaemia and 3 of pediatric T-ALL. *Aim.* To characterize TAF_I-NUP214 fusion detected by FISH and molecular studies in 4 adults with pre-T-ALL and cryptic del(9)(q34), and to establish the incidence of TAF_I-NUP214 in adult T-ALL. *Methods.* PCR and cloning experiments used TAF540ex6F + NUP2916ex21R primers for the first amplification round and TAF747ex7F + NUP2601ex19R for nested PCR. Isoform-specific PCR was also performed using NUP2916ex21R as reverse primer, TAFIa_42F for the TAF-I alpha isoform, and TAFIb_38F for TAF-I beta.

Reverse primer NUP2601ex19R and TAFIa_42F or TAFIb_38F were used for nested PCR. Gene expression profiling studies used HGU133 plus 2.0 gene chips according to Affymetrix protocols. Multiplex RT-PCR used TAF_540ex6F and NUP_2916ex21R primers for the first reaction step and TAF747ex7F and NUP2601ex19R for the second. *Results.* The first round PCR detected a 802 bp product in patient 1 and a 643 bp product in the others; the second round of amplification detected a 280 bp product in patient 1 and a 121 bp product in the others. Sequence analysis revealed TAFI nucleotide 813 (exon 7) was fused to NUP214 nucleotide 2389 (exon 17) in patient 1 and to nucleotide 2548 (exon18) in patients 2, 3 and 4. Moreover, both TAF_Ialpha-NUP214 and TAF_Ibeta-NUP214 isoforms were detected in all 4 cases. Gene expression profiling revealed up-regulation of a set of HOXA gene and down-modulation of FNBP1, C9orf78, and USP20 genes, all mapping at 9q34.11, the region undergoing cryptic deletion. *Conclusions.* TAF_I-NUP214 is new recurrent molecular lesion in a subset of adult T-ALL showing an incidence of 3.6 % in a series of 110 cases. In our cohort of patients TAF_I-NUP214 correlates with pre-T phenotype and is characterized by over-expression of HOXA genes, a signature already described in T-ALL with MLL-translocations, CALM-AF10, and inv(7)/HOXA. *Acknowledgments.* MIUR (Ministero per l'istruzione, l'università e la ricerca scientifica), Italy

P114

P2 RECEPTORS ARE EXPRESSED ON ACUTE MYELOBLASTIC LEUKEMIA CELLS AND THEIR STIMULATION AFFECTS LEUKEMIA CELLS FUNCTION

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Extracellular nucleotides ATP and UTP are emerging as ubiquitous molecules involved in a wide variety of biological responses, including cell proliferation and differentiation as well as cell death (by apoptosis or necrosis). The biological effects induced by extracellular nucleotides in many cell types are mediated by specific plasma membrane receptors, named P2 receptors (P2R). Previous data from our group have demonstrated the expression of P2R in normal stem cells and the capacity of extracellular nucleotides to induce their proliferation, migration and engraftment to injured bone marrow. In this study, we assessed whether P2R are expressed on leukemic cells and whether their engagement modifies acute leukemic cell (AML) functions. Western blot analyses showed expression of the following P2X and P2Y subtypes: P2X4, P2X7, P2Y1. In cells of eight AML subjects out of nine, intensity of the P2X4 band was much more pronounced than in $CD34^+$ control cells. Whereas expression of P2X7 and P2Y11 differed among AML patients. At the functional level, stimulation of AML cells by extracellular nucleotides (ATP, UTP, BzATP) induced intracellular Ca^{2+} concentration increases. Since Ca2+ signalling is known to be involved in cell proliferation and differentiation, experiments on AML proliferation in the presence of nucleotides were performed. The total colony formation potential of blasts was inhibited by the addition of 1 nM ATP (30% mean reduction) whereas UTP did not affect the clonogenic capacity. A more profound effect was exerted by the stable analogs INS415 and INS973, their addition to the culture medium, at the concentation 500 nM, induced 85% and 50% reduction of the clonogenic growth of AML cells, respectively. We observed a pronounced effect of triphosphate nucleotides on blast migration. ATP and UTP acted as chemotactic agents and stimulated blast migration in response to CXCL12. In summary, our data demonstrate that P2R are expressed and functional in AML cells. Engagement of P2R by extracellular nucleotides exert opposite effects on leukemic cells proliferation as compared to their normal counterparts. Characterization of P2 receptors expression and function in AML may help in understading the mechanism of aberrant differentiation of neoplastic cells and tumor progression.

P115

IN VITRO VALPROIC ACID TREATMENT MODIFIES THE PROTEOME OF AML1/ETO POSITIVE LEUKEMIA CELLS

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Alterations in chromatin organization are a shared mechanism of leukemogenesis. Drugs affecting epigenetic modulation of gene expression have been proposed in clinics. Our starting hypothesis is that CBF-AMLs, recruiting to DNA HDAC/DNMT repressor protein complex, could be specifically sensitive to histone deacetylase inhibitors (HDACi). Valproic acid (VPA) is an oral HDACi, available for treatment in Europe. We analysed the effects of the HDAC inhibitor VPA 2 mM on the proteome of AML1-ETO-positive, AML blast cells. We also analysed the differences in response to VPA shown by AML1/ETO-inducible U937-A/E-9/14/18 cells. Total cell protein extracts of treated and untreated AML1-ETO-positive cells were separated by two dimensional electrophoresis (2DE) on pH non linear gradient 3-10. The 2D gels were analysed by adequate software (Image Master TM Platinum), for spot detection and quantification. 2D gels were virtually superimposed and aligned for proteomic comparison. Significant differences both qualitative and quantitative in protein spots appeared between VPA treated (24-72 hours) and untreated cells. Moreover, spot differences were increasing throughout culture, as well as acetylation, inhibition of cell proliferation and apoptosis. MS identified proteins with different level of expression in treated *vs.* untreated cells. MALDI-TOF MS analysis (PMF and/or MS/MS exp) of the most representative protein spots indicated that identified proteins could be divided into 3 functional categories: DNA binding/signal transduction proteins, metabolic enzymes, heat shock proteins and chaperones. Proteins induced by VPA were: enolase-1, laminin-binding protein, Siah-interacting protein(SIP). VPA significantly enhanced the expression of the following proteins: nucleophosmin, EEF1D protein, stathmin 1, activator of HSP-90 ATPasi, tumor rejection antigen (gp 96).VPA inhibited the expression of cofilin-1 and calreticulin precursor variant, proteosome subunit alpha and beta. Additional proteins had different spot distribution in VPA treated cells *vs.* untreated, indicating post translational modifications induced by the HDAC inhibitor. Western blots with specific antibodies for single identified proteins were performed to confirm quantitative and qualitative results. Better characterization of the significance of each of these proteins in AML1-ETO positive cell proliferation and survival could be important to identify mechanisms of sensitivity to HDAC inhibitors.

P116

SERUM FERRITIN AS PROGNOSTIC MARKER OF RESPONSE IN ADULT ACUTE MYELOID LEUKEMIA

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Background. Recent studies have suggested a link between iron overload and posttransplantation liver toxicity, infectious susceptibility, and even survival in patients undergoing hematopoietic stem cell transplantation for hematological malignancies. *Aims.* To date, there are no data to show that the availability of iron can and does play a critical role in adult acute myeloid leukemia (AML). We report here a study to determine the role as prognostic factor of pre-treatment serum ferritin in adult AML. *Methods.* We studied 30 consecutive adult de novo AML patients. For each case included in this study, serum ferritin level was determined at the onset of the disease. The median age of patients (15 males and 15 females) was 57 years (ranged from 16 y.rs to 75 y.rs); according to the FAB criteria the subtypes were: 1 M0, 3 M1, 14 M2, 3 M3, 5 M4, 3 M5b, 1 M6. All cases were treated with standard induction therapy. Student's t-test or the Mann-Whitney test was performed for comparisons of means. A 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 it was calculated using the Kaplan-Meier method; the log-rank test was used to compare survival curves. Only p values <0.05 were considered to be statistically significant. The AML patients were subdivided in two groups according to ferritin serum value (<800 versus > 800 ng/mL). Results. Fourteen (47%) patients showed a ferritin serum value > 800 ng/mL. Compared with < 800 ng/mL group,

patients with serum ferritin > 800 ng/mL were more frequently non responders to chemotherapy treatment (57% *vs.* 25%, p=0.009) and they had shorter OS (42 days *vs.* the median survival not reached for the < 800 group, p=0.001). However, after excluding the M3 patients from the analysis, the differences between the two groups remained (Figure 1). Moreover, patients with serum ferritin > 800 ng/mL showed a trend for a higher frequency of documented infections during induction treatment (36% *vs.* 6%, p=0.07). It is noterworthy that the patients in complete remission presented at the onset of AML a median ferritin value lower to that associated to the non responders patients (483 ng/mL versus 1233 ng/mL, p=0.03). *Conclusions.* It is well-known the role of iron metabolism in sepsis and carcinogenesis. The results of our study demonstrated a link between ferritin serum and AML prognosis. Further studies will be required in a large series of AML patients to confirm the useful of serum ferritin as prognostic marker.

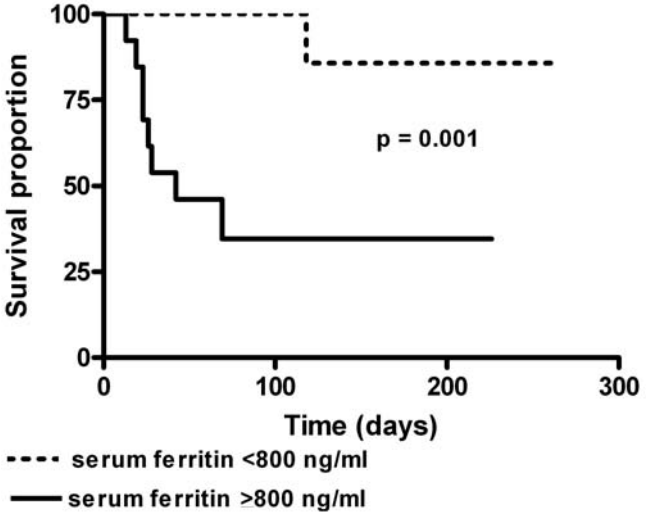

Figure 1.

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FLUDARABINE-BASED INDUCTION THERAPY DOES NOT OVERCOME THE NEGATIVE EFFECT OF BCRP (ABCG2) OVEREXPRESSION ON REMISSION DURATION IN ADULT DE NOVO ACUTE MYELOID LEUKEMIA PATIENTS

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Background. Breast cancer resistance protein (BCRP), a recently identified multidrug resistance (MDR) protein of the ATP-binding cassette family, is associated with a shorter disease free survival (DFS) in AML patients. Fludarabine – based induction therapy displayed interesting results in AML, with high rates of complete remission (CR), and has been shown to overcome the negative effect of P-glycoprotein (PGP), another MDR-related protein, over-expression. No data are available about Fludarabine effect on remission duration in AML patients with BCRP over-expression. *Aims.* We measured the levels of BCRP in 124 cases of AML, treated with a Fludarabine-based induction therapy, to evaluate the possible effect of this regimen on DFS according to BCRP expression. *Methods.* One hundred and twenty-four patients with a diagnosis of *de novo* AML were included in our study. Cases with PML/RAR-alpha rearrangement were excluded. There were 62 males (50%) and 62 females, with a median age of 56 years (range: 16-79). All patients were treated with an induction regimen containing fludarabine, cytarabine and idarubicin, and a consolidation course with high-dose cytarabine and idarubicin. BCRP expression was measured at diagnosis with flow cytometric analysis. *Results.* BCRP protein was over-expressed in 55/124 (44%) patients. BCRP positive cases showed a higher WBC count (p<0.01) and PGP-over-expression (p=0.04) compared to BCRP negative patients, while no statistically significant differences were seen according to age, karyotype and CD34 expression between BCRP-positive and BCRP-negative cases. CR was obtained in 71/122 (57%) patients. Advanced age and high WBC counts affected remission rate. As expected, BCRP expression was not associated with CR obtainment but a significantly shorter DFS was observed in patients with high BCRP expression (8 *vs.* 16 months; p=0.02). A shorter survival was associated with response to induction therapy (CR or not), advanced age and CD34 positivity. Conclusions. BCRP over-expression did not influence achievement of CR in Fludarabine-treated patients, but significantly affected CR duration, as BCRP-positive patients displayed a shorter disease free survival. Fludarabine does not overcome BCRP negative effect on DFS, and AML cases with BCRP over-expression could take advantage of an intensive post-remission therapy.

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PLATELET GEL FOR HEALING SINUS PILONIDALIS IN PATIENT WITH ACUTE LYMPHOBLASTIC LEUKEMIA IN CHEMOTHERAPY

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Background. Topical use of platelet gel (PG) is a relatively new technology which may stimulate and accelerate soft-tissue and bone healing; it offers opportunities for treatment of wounds, ulcers, soft-tissues injuries and various other applications in regenerative medicine. The rational to employ this technique is to mimic physiological wound healing and reparative processes. Tissue repair begins with clot formation and platelet degranulation, which release the growth factors (GFs) necessary for wound repair (PDGF, TGF-beta, IGF-I, IGF-II, EGF, FGF-beta). Platelet-derived GFs are biologically active substances that enhance tissue repair mechanisms such as chemotaxis, cell proliferation, angiogenesis extracellular matrix deposition and remodeling. Aim. Effectiveness of local homologous PG for healing sinus pilonidalis as a non-invasive method alternative to surgery. *Methods.* A nine year old young female undergoing chemotherapy for acute lymphoblastic leukemia relapsed after hemopoietic stem cell transplantation developed two septic episodes due to Gram-negative bacteria (multiple antibiotic-resistant Escherichia Coli) during the neutropenic period likely from sinus pilonidalis. The sinus pilonidalis was firstly sterilized with topical disinfection and systemic antibiotics. The therapeutic protocol consisted of the once-weekly local application of homologous PG for 4 weeks as follows: 1st day local PG application; 4th day removal a PG and thereafter daily medication with idrogel. *Results.* The pain reduced after the first PG application and sinus pilonidalis completely resolved (Figure 1) after 4 weeks. The patient continued chemotherapy without local and/or systemic bacterial infections. Conclusions. The local application of PG may represent a valuable alternative to surgery. Moreover, the rapid healing achieved with local application of PG allows a reduction in the risk of secondary infection, pain, and need for hospitalization, as well as in the improvement of patient quality of life.

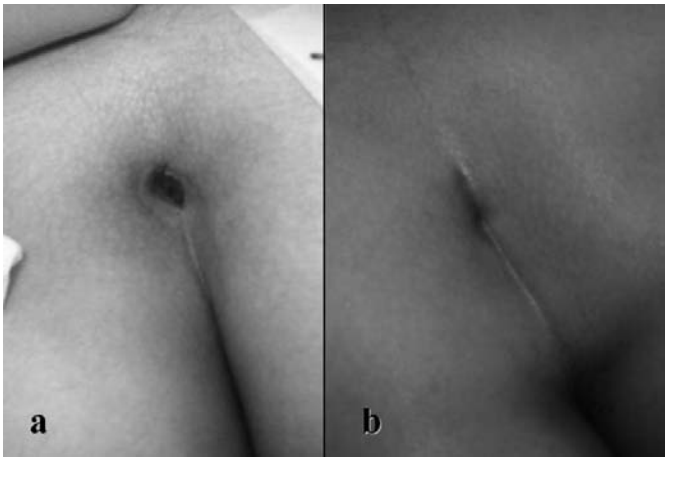

Figure 1: a) at beginning b) after 4 weeks.

Acute Leukemias (III)

P119

IN VITRO AZACITIDINE TREATMENT MODIFIES THE PROTEOME OF AML1/ETO POSITIVE LEUKEMIA CELLS

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Alterations in chromatin organization are a shared mechanism of leukemogenesis. Drugs affecting epigenetic modulation of gene expression have been proposed in clinics successfully. Our starting hypothesis is that CBF-AMLs, could be specifically sensitive to DNAMethyltransferase inhibitors(DNMTi). We analysed the effects of the DNMTi-AZA on the proteome of AML1-ETO positive, AML blast cells and the differences in response to AZA shown by AML1/ETO-inducible U937-A/E-9/14/18 cells. Total cell protein extracts of treated and untreated AML1-ETO-positive cells were separated by two dimensional electrophoresis (2DE) on non-linear pH gradient 3-10. The 2D gels were analysed by adequate software (Image Master TM Platinum),for spot detection and quantification. 2D gels were virtually superimposed and aligned for proteomic comparison. Methylation of p15 promoter (methylation specific-PCR), histone acetylation (Western blot), inhibition of proliferation (cell cycle assay), and apoptosis (annexin–V assay) were evaluated in parallel. AZA inhibited cell proliferation and induced significant levels of apoptosis in AML1-ETO expressing cells. At proteome analysis, significant differences both qualitative and quantitative in protein spots appeared between AZA treated and untreated cells. Total spots number was 620 in untreated cells, and 486 in AZA treated. 400 spots were matching, and MS identified 31 proteins with different level of expression in treated *vs.* untreated cells. MALDI-TOF MS analysis (PMF and/or MS/MS exp) indicated that identified proteins could be divided into 4 groups: DNA binding/signal transduction proteins, metabolic enzymes, heat shock proteins and chaperones, structural proteins. AZA induced calmodulin and Cypa/Hvgpia proteins and significantly enhanced the expression of NM23a, hPCNA, both DNA binding proteins and the structural protein muscle Z-line. AZA inhibited the expression of ER60 protease, VCP protein, proline-4-hydroxylase. Heat shock proteins like HSP90, 60, 70, gp96, and tubulin and enolase 1 had different spot distribution in AZA treated lysates *vs.* untreated, indicating post translational modifications induced by the hypomethylating agent. Western blots with specific antibodies were performed to confirm quantitative and qualitative results. We observed a significant modulation by AZA of proteins regulating cell trafficking and of chaperone proteins. Their activity could be important in identifying mechanisms of sensitivity to DNMTi.

P120

CXCR4 AS A PREDICTOR OF RESPONSE IN ACUTE MYELOID LEUKEMIA

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The expression of CXCR4 (CD184) has been associated with poor prognosis in Acute Myeloid Leukemia (AML) and it has also been suggested that the CXCL12(SDF-1a)/CXCR4 interaction contributes to the resistance of leukemia cells to chemotherapy-induced apoptosis. Inhibition of CXCR4 was found to enhance chemotherapy-induced apoptosis in a subset of leukemic myeloblasts that carry Flt3 mutations and to overcome chemoresistance associated with stromal activity. NPM variants with a cytoplasmic localization represent the most common mutation detected in myeloid malignancies and are associated with a favourable clinical outcome. A recent study provides biological evidence for a novel role for NPM as a negative regulator of CXCR4 signalling induced by CXCL12: suppression of NPM expression enhanced chemotactic responses to CXCL12, and conversely, over-expression of a cytosolic NPM mutant reduced chemotaxis induced by CXCL12. We investigated whether CD184 expression is a negative predictor factor for response to chemotherapy and if there is clinical evidence that NPM mutations could overcome chemoresistance to induction therapy in this subset of patients. The expression of CD184 was analyzed by flow cytometric methods in a group of 64 cases of adult AML at onset of disease, diagnosed at our Institution since January 2006. The diagnosis was performed according to FAB/WHO criteria; all patients received intensive chemotherapy according to institutional protocols. There were 32 males and 32 females and median age was 46 years (range 18-65). AML cells were gated based upon their CD45 expression and samples were considered positive if CD184 was expressed by more than 20% of blasts. CD184 was positive in 42 and negative in 22 cases. There was no significant difference between the two groups in terms of sex, age, Hb level, WBC and Plt counts, percentage of blasts, and occurrence of the NPM mutation. The CR rate was 45% in $CD184^+$ and 82% in $CD184^-$ (p=0.03); among $CD184^+$ cases, the CR rate was significantly higher in $NPMc^+$ cases, (p=0.03). Our results show that CD184 expression is associated with a lower rate of CR after induction therapy and this association is stronger in NPM unmutated cases, suggesting that CD184 expression is a negative predictive factor for response to chemotherapy. Further data are needed to verify if the biological role of the cytosolic NPM mutant as a negative regulator of CXCR4 signalling induced by CXCL12 could have a clinical role contributing to overcome the resistance of leukemic cells to induction chemotherapy.

P121

THE DYNAMICS OF ABL MUTATIONS ONSET IN PH+ ACUTE LYMPHOBLASTIC LEUKEMIA TREATED WITH IMATINIB MESYLATE

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The Philadelphia chromosome-positive (Ph^+) is a genetic marker for a dismal prognosis subgroup of acute lymphoid leukemia (ALL) patients. Treatment with conventional chemotherapy has not substantially improved their long-term outcomes and despite its efficacy, Imatinib (Ima) treatment may induce specific resistance in a proportion of patients, mainly because of the occurrence of ABL mutations. Acquired Ima resistance in Ph^+ ALL has been associated with a small number of mutations at the kinase domain of BCR-ABL. In this study, we evaluated the dynamics of mutations before and during Ima treatment in 37 patients with Ph^+ ALL enrolled into the GIMEMA LAL2000 protocol. In this protocol, patients who obtain complete remission after conventional induction and consolidation treatment, receive Ima (400 mg/d x2) as maintenance therapy. Mutation analysis was performed by DHPLC analysis in all patients at diagnosis, after consolidation chemotherapy, at three-month intervals during Ima maintenance and at relapse. Mutation detection sensitivity of technique was 10%. At diagnosis, we found four low-titer ABL mutations: two of these were silent mutations, i.e. without amino acid substitution (nt. 259 and 320), and were not longer detectable at subsequent evaluations. After consolidation treatment despite in remission, 8 patients showed a mutation at Abl gene, three of which were silent mutation. Interestingly, one of the patient with silent mutation also had a transient, low titer G259G silent mutation at diagnosis. Subsequent follow-up analysis, showed the presence of low-titer ABL mutations in 6 of 14 patients (43%) who maintained remission at the end of study and in 13 of 20 (65%) who relapsed. Among the 13 patients who showed mutations at relapse, 11 had the same mutation already shown during follow-up, but two had a different mutation in the Ph^+ cells of the relapse. Noteworthy, in 3 of the 6 mutated patients who maintained remission by the end of follow-up, mutations were not longer detected during follow-up. Taken together, these data indicate that ABL mutations represent one of the most common cause of Ima resistance, but their presence seem to be per se insufficient to induce resistance since the high rate of transient mutation detected in ima treated and not treated patients

P122

PRECLINICAL DEVELOPMENT OF MEK INHIBITION-BASED THERAPEUTIC STRATEGIES IN ACUTE MYELOID LEUKEMIA

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We have demonstrated that selective MEK inhibitors (MEK-I) (i.e. PD98059, CI-1040, etc.) potently inhibit the growth of AML cell lines and ex vivo-cultured primary AML blasts. However, these effects are mostly related to the inhibition of cell cycle progression, while apoptosis induction requires higher concentrations of the inhibitors and longer times of exposure. Thus, we investigated MEK-I-induced changes in phospho-protein expression and gene expression profile, in order to identify relevant downstream targets and to design rational MEK-I-based combination strategies with synergistic anti-leukemic potential. Analysis of phosphorylation levels of 18 different target proteins performed in OCI-AML3 cells indicated that MEK blockade induces, among other effects, an over-activation of RAF and MEK, suggesting the interruption of a negative feedback loop; moreover, gene expression profiling indicated upregulation of the Flt-3 receptor. Based on these observations, as well as on recent evidence indicating that the Raf inhibitor sorafenib directly inhibits signaling through Flt-3, experiments were performed in OCI-AML3 and MOLM-13 (which harbors a Flt3 ITD) cells to test the activity of MEK-I in combination with sorafenib. Preliminary results indicate that the combination may indeed result in synergistic growth-inhibitory and pro-apoptotic activity of the two inhibitors with combination indexes (CI), as defined using isobologram analysis (Chou–Talalay method), ranging from 0.003 to 0.6 in OCI-AML3 and MOLM-13 cells after 72h. Since we demonstrated the ability of MEK-I to modulate the expression, among others, of genes controlling mitochondrial homeostasis (e.g. PPIF, GRPEL1), we next investigated the impact of a simultaneous inhibition of the MEK and bcl-2 pathways in AML cells. Preliminary results obtained exposing OCI-AML3 and MOLM-13 cells to a combination of MEK-I and the bcl-2/bcl-xL inhibitor ABT-737 (kindly provided by Abbott Laboratories), again indicate a synergistic growth-inhibitory and pro-apoptotic activity of the these two small molecules, with CI <0.5 in both cellular models after 72h. Overall these results support the role of the Raf/MEK/ERK kinase module as a prime target for the molecular therapy of AML and highlight the concept that simultaneous targeting of multiple signaling pathways may induce highly synergistic anti-leukemic effects.

P123

STUDY OF WT-1 EXPRESSION AND ANALYSIS OF JH REARRANGEMENT MAY PREDICT RISK OF RELAPSE IN ADULTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA SUBMITTED TO ALLOGENEIC BMT

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The study of the monoclonal JH rearrangement is widely applied in the minimal residual disease (MRD) analysis of chronic and acute B-lymphoproliferative diseases, when a specific intrinsic marker is lacking. Patients who are JH positive at the end of therapy do not necessarily have an increased risk for relapse. Wilms' tumor gene (WT1) is a tumor suppressor gene involved in the regulation of cell growth and differentiation. WT1 transcripts and nuclear protein have been detected in the majority of human acute leukemias. The level of WT1 expression has been associated with the presence, persistence or reappearance of a leukemic clone. A significant association between WT1 level and prognosis has been shown in patients with acute myeloid leukaemia and acute lymphoblastic leukaemia (ALL) receiving chemotherapy. We analyzed both WT1 expression and JH rearrangement in 32 adult ALL patients submitted to allogeneic BMT to investigate whether the sequential monitoring of both markers is able to predict the outcome and improve the disease management. JH assay was performed by fingerprint method and WT1 expression by Real-time PCR , normalized on Abl expression. In this scenario WT1 expression could reflect a functional state of leukemic clone and predict relapse.14 males and 18 females, with a median age of 35 years (range, 19 to 51) have been studied . Ten patients showed t(9;22), 2 t(4;11), 1 a complex karyotype; 17 patients had a normal karyotype. All patient had received a conventional induction and consolidation therapy. Molecular study was performed before BMT, at day +100, and then every 3 months. In all the patients one or more JH monoclonal bands had been identified on the marrow sample studied at diagnosis .In the pre-BMT samples JH rearrangements have been detected in all the 32 patients, whereas WT1 expression was shown in 15 patients. In 3 patients in complete hematologic remission the high level of WT1 expression was predictive of an early relapse. At day +100 post BMT 9 patients were JH negative: 7 of these patients are alive and disease free, 2 have died, due to extensive GVHD, in complete remission. All these patients had normal levels of WT 1 expression. 23 patients were JH positive: 7 had high level (≥100) of WT1 and experienced an early relapse, 2 had intermediate level (50-100) and both relapsed in extramedullary site, 14 patients had normal level of WT1 (<50) and maintain CR with a median follow up of 24 months. Nine of these 13 received donor lymphocyte infusions for persisting JH positive molecular status: 4 of them developed cGVHD and one patient died of extensive cGVHD. In conclusion, our data suggest that elevated WT 1 level post BMT (day +100) may have an unfavorable prognostic value. JH negative status post BMT is always associated with a low level of WT1 expression and is associated with a very low risk of disease recurrence.

P124

INCREASING BCR-ABL EXPRESSION LEVELS AND/OR OCCURRENCE OF ABL POINT MUTATIONS NOT ALWAYS PREDICT RESISTANCE TO IMATINIB MESYLATE IN BCR-ABL POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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Imatinib Mesylate (IM) has significantly improved response rates in patients affected by Philadelphia positive (Ph^+) acute lymphoblastic leukemia (ALL). However, relapse occurs in 20-50% of patients and the development of point mutations in the ABL gene may be the predominant cause. The current monitoring of minimal residual disease (MRD) in Ph^+ ALL patients includes the Q-RT-PCR assessment of BCR-ABL expression and the screening for ABL mutations but it is still to be defined the clinical value of these methods in predicting the occurrence of clinical relapse. Therefore, we report the case of an adult Ph^+ ALL patient, maintained with IM alone, in which levels of BCR-ABL were compared to search for ABL mutations to monitor MRD. Our patient was 44 years old at the time of diagnosis, on July 2002. After a good response to chemotherapy (based on the GIMEMA LAL 2000 protocol), he was enrolled in the GIMEMA 0201-A protocol, which contemplates a post-consolidation therapy with IM 800 mg/die for six months. This treatment was continued till to date, because the patient refused HSCT. The patient is actually in first complete haematological remission. Q-RT-PCR was performed at diagnosis and at prefixed time during the follow-up, according to the methods developed by the European Concerted Action of the Europe Against Cancer Program. Search for ABL point mutations was performed using DHPLC and direct sequencing.After chemotherapy the patient achieved a 3 log reduction of the BCR-ABL/ABL ratio respect to diagnostic value. During IM, the BCR-ABL/ABL ratio fluctuated around these low values, becoming undetectable in more than one sample. However, a 2 log increase in the BCR-ABL expression was observed in two different periods. A concomitant mutation Q300R was detected in only one of these samples (Figure 1). Continuing of IM maintain HCR and induced both a decrease in the BCR-ABL expression and the disappearance of Q300R mutation. To date, Q300R has never been observed in patients with Ph^+ leukaemia. Rarely, Q300H, a variant of this substitution, able to provide a moderate resistance to IM, has been reported. In conclusion, our data suggest that the occasionally increase in the BCR-ABL levels and the occurrence

of ABL point mutations are not always related to the occurrence of clinical resistance to IM. Further studies are needed to address this issue, that could be of paramount relevance in the management of Ph+ ALL patients receiving tyrosine kinase inhibitors.

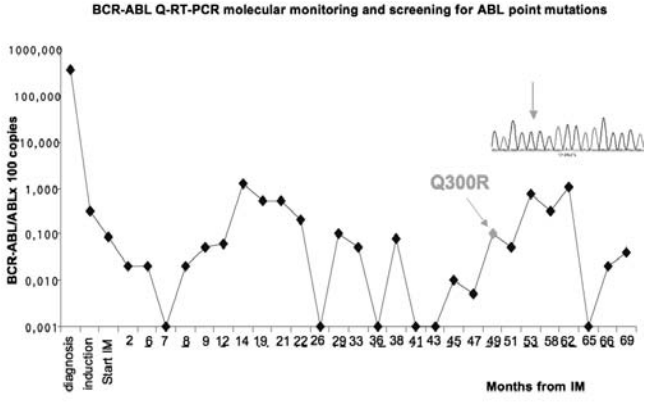

Figure 1. Q-RT-PCR molecular monitoring of patient MRD from diagnosis and during the follow-up. Analysis of ABL point mutations revealed in one sample the presence of a complete nucleotide substitution of CAG (cytosine, adenine, guanine) to CGG (cytosine, guanine, guanine) in position 1047, leading to an amino acid variation from glutamine to arginine at residue 300 (Q300R). The subsequent samples showed a wild-type sequence with the complete disappearance of the mutation.

P125

INTERIM ANALYSIS OF A PHASE I/II STUDY OF TIPIFARNIB AND BORTEZOMIB IN THE TREATMENT OF POOR RISK ADULT ACUTE MYELOID LEUKEMIA

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Background. Outcome of elderly acute myeloid leukemia (AML) patients is dismal. Tipifarnib (Zarnestra, Z) and Bortezomib (Velcade,V) are new, targeted-treatments that might improve current results. In particular, Z is a farnesyltransferase inhibitor effective in AML, allowing complete remission (CR) rates ranging from 8 to 22%. Notably, the response rate appeared superior among patients with higher RASGRP1/APTX gene expression ratio. V is a proteasome inhibitor, possibly effective in AML as for phase I studies. Interestingly, Z and V appeared synergistic in AML cell lines. *Aim.* We designed a phase I/II study aiming to assess the maximum tolerated dose (MTD) and dose-limiting toxicity (DLT) of V in association with standard doses of Z and the efficacy and toxicity of the combination, in AML patients >18 years, unfit for conventional therapy, or >60 years, in relapse. Furthermore, we aimed to assess the correlation between RASGRP1/APTX ratio and treatment response. Methods. V was administered as weekly infusion for three consecutive weeks (days 1, 8, 15), starting from 0.7 mg/m^2 and increasing by 0.3 mg/m^2 until the DLT was reached. Z was administered at the daily dose of 600 mg BID for 21 consecutive days. Response was assessed at the end of each cycle (28 days). Patients' withdrawn was planned in case of progression or stable disease after six cycles. Real-time quantitative PCR (q-PCR) was used for RASGRP1/APTX genes evaluation. *Results.* 45 patients were enrolled: 12 in the phase I and 33 in the phase II. 11/12 patients were evaluable in the phase I. Five patients received V at the dose of 0.7 mg/m^2 without reporting DLT. Conversely, DLT was reached at the dose of 1.0 mg/m^2 due to grade III SNC toxicity, recorded in 1/6 patients. 22/45 patients were evaluable for treatment response; transient reduction of peripheral blasts (>50% to baseline) was observed in 20/22 cases. 2 patients achieved CR and 1 obtained a partial response (PR). 1 patient had a haematological improvement (HI), 12/22 had a stable disease (SD) and 5/22 showed progressive disease (PD). Interestingly, the 4 responders (CR+PR+HI) had a significant higher RASGRP1/APTX ratio respect to non responder (p=0.017). *Conclusion.* We conclude that the MTD of V in association with Z is 1.0 mg/m^2. The association seemed to be safe, with response rate (18%) similar to what reported for Z alone. Finally, though in few cases, RASGPR1/APTX ratio was confirmed to be associated to treatment response.

P126

DISULFIRAM INDUCES APOPTOSIS IN HUMAN AML AND ALL CELL

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Acute mieloid and lymphoblastic leukemia (AML and ALL) are malignant diseases characterized by abnormal proliferation of clonal precursor cells. Proteasome is a novel, interesting target in cancer drug therapy, and the proteasome inhibitor Bortezomib has been used for its antitumor activity in multiple myeloma and other lymphoid malignancies. The thiocarbamate alcohol-abuse deterrent disulfiram (DSF) is an aldehyde dehydrogenase (ALDH) inhibitor with documented proteasome-inhibiting activity. It was previously shown to block the P-glycoprotein extrusion pump, to inhibit the transcription factor nuclear factor-kappaB (NF-kB), to sensitize tumors to chemotherapy, to reduce angiogenesis, and to inhibit tumor growth in mice. Here we show that AML (12 samples) and ALL (5 samples) primary cells from newly diagnosed patients were significantly sensitive to DSF (DSF high dose induces about 60-70% of cell death after 48 hours of treatment). These cells are ALDH weakly positive. When in combination with CuSO4, DSF has a cytotoxic effect *in vitro* at lower concentrations than disulfiram alone (DSF low dose plus CuSO4 induces about 80-90% of cell death after 48 hours of treatment). The apoptotic effect was comparable to that exert by therapy with Cytarabine, Etoposide, Daunorubicine alone or in combination for AML samples or Vincristine, Desametazone and Daunorubicine alone or in combination for ALL samples. Conversely, DSF had only a weak effect on normal CD34 and peripheral blood mononuclear cells (<30% of cell death). In addition we show that DSF plus CuSO4 induce loss of mithocondrial membrane potential thus suggesting the involvement of mithocondrial apoptotic pathway. These results may suggest a novel strategy for treating leukemia by employing an old drug, with known side-effect, toward a new therapeutic use.

P127

MYELOID INTO LYMPHOID NEOPLASM: A CASE OF ACUTE MYELOID LEUKEMIA DEVELOPED IN UNTREATED LYMPHOPLASMACYTOID LYMPHOMA

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On May 2006 a mild thrombocytopenia (100000/microL) and monoclonal component (MC) IgM k at the serum protein electrophoresis were detected in a 67-year old man with an unremarkable medical history. Serum IgM were 13.1 g/L. Other laboratory tests were normal, Bence-Jones urinary protein absent. Physical and ultrasound examination showed neither hepato-splenomegaly nor lymphadenopathies. The bone marrow (BM) trephine biopsy demonstrated non Hodgkin's lymphoma (NHL) derived from B peripheral lymphocytes, lymphoplasmacytoid type. Molecular biology and karyotype analysis were normal. On February 2008 an increase of MC (IgM 31.3 g/L) occurred, with mild anemia and unchanged thrombocytopenia, without leukocytosis, so the patient underwent disease revaluation for suspected progression. Unexpectedly BM aspiration showed 90% of blasts of monocytic morphology, immunophenotype confirming blasts CD33$^+$, CD13$^+$, CD34$^-$, HLA-DR$^+$, CD15$^+$, CD64$^+$, CD68$^+$, CD3$^-$, CD4$^+$, CD11b$^+$, MPO weakly$^+$. The immunological pattern was consistent with monoblastic acute leukemia (AML) with maturation. Karyotype resulted normal, molecular biology revealed no evidence of chromosomal translocations among BCR/ABL, AML1/ETO, CBFbeta/MYH11, DEK/CAN, PML-RARalpha. BM trephine biopsy confirmed AML M5b, showing a residual NHL, as resulting form CD20$^+$ immunohistochemistry. The patient underwent polychemotherapy according to FLAN modified schedule (Fludarabine 30 mg/mq and Mitoxantrone 6 mg/mq d 1-3, Cytarabine 1 g/mq d 1-5). BM aspiration after induction therapy revealed complete remission and serum IgM decreased to 18.8 g/L. The patient is now undergoing a second course of chemotherapy. Few cases of low-grade NHL and concomitant AML have been described, especially regarding therapy-related secondary AML. In this case a patient presented at first a lymphoplasmacytic lymphoma, then he developed a myeloid leukaemia. An accidental occurrence of independent haematological malignancies is unlikely because of the rarity of the two conditions. Both diseases may originate as the result of neoplastic transformation of a common precursor, differentiating into two different cell lines. The secondary appearance of an AML in a patient affected by a lymphoid neoplasm could reflect a reduced immunocompetence and/or antioncogene suppression leading to the expansion of multiple neoplastic clones. Chemotherapy with a fludarabine-based scheme was preferred, because of its citotoxic effect on both myeloid and lymphoid malignancies.

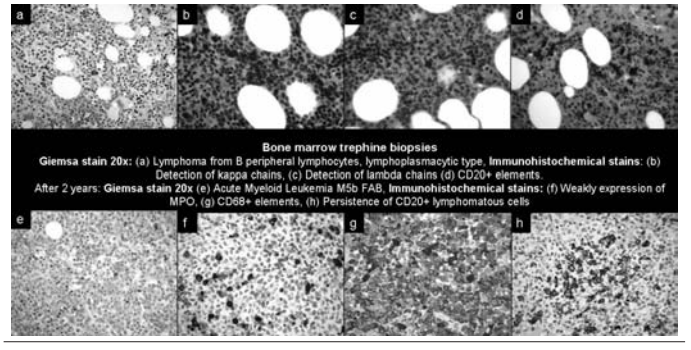

Figure 1.

Chronic Lymphocytic Leukemia (I)

P128

GENE EXPRESSION PROFILING OF CD38+CD49D+ CHRONIC LYMPHOCYTIC LEUKEMIA CELLS: ROLE OF THE CHEMOKINES CCL3 AND CCL4 IN THE CROSS-TALK BETWEEN CLL CELLS AND TUMOR MICROENVIRONMENT

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We and others identified CD49d as an independent negative prognostic marker in chronic lymphocytic leukemia (CLL), whose expression was strictly correlated to that of the other negative prognosticator CD38. To find a link between CD38 and CD49d that may explain their negative impact in CLL, we performed gene expression profiling (GEP) studies utilizing purified CLL cells from 11 CD38$^+$CD49$^+$ and 15 CD38$^-$CD49d$^-$ CLL. Among 205 genes up-regulated in CD38$^+$CD49d$^+$ CLL cells, we found the C-C chemokines CCL3 (median-log difference, MLD=1.79) and CCL4 (MLD=2.1), whose over-expression was confirmed by real-time quantitative PCR (RTQ-PCR). *In vitro* experiments, performed on purified tumor cells from CD38$^+$CD49d$^+$ CLL cases cultured for 14 and 24 hours (t14 and t24) in the presence of either the agonist anti-CD38 monoclonal antibody (mAb) IB4 or the non-agonistic anti-CD38 mAb IB6 as control, demonstrated upregulation of CCL3/CCL4 transcripts at t14 (CCL3: mean fold increase=18, p=0.041; CCL4: mean fold increase=13.8, p=0.005), as assessed by RTQ-PCR, associated with an increased release of CCL3/CCL4 proteins at t24 (CCL3: mean =0.9 ng/mL, mean fold increase=14, p=0.003; CCL4: mean =1.7 ng/mL, mean fold increase=49, p=0.01), as assessed by ELISA. Consistently, CCL3 was detected by immunohistochemistry (IHC) in neoplastic cells from bone marrow biopsies (BMBs) of CD38+CD49d+ but not from CD38-CD49d- CLL. High expression of the CCL3/CCL4 specific receptors CCR1 and CCR5 was found on CLL-derived monocytes/macrophages. Accordingly, a higher number of infiltrating CD68+ macrophages was found in BMBs of CD38$^+$CD49d$^+$ as compared to CD38$^-$CD49d$^-$ CLLs (p=0.013), suggesting a role of these cells as target for CLL-derived chemokines. In parallel experiments, conditioned media from CCL3-stimulated macrophage cultures were demonstrated to induce expression of the CD49d-ligand vascular cell adhesion molecule-1 (VCAM-1) by human endothelial cell cultures. Again, IHC analysis of CLL BMBs showed a meshwork of VCAM-1-positive cells in CD38$^+$CD49d$^+$ but not in CD38$^-$CD49d$^-$ CLL infiltrates (p=0.002). Preliminary ELISA experiments indicated TNF-alpha among the major cytokines involved in VCAM-1 up-regulation by endothelial cells. Altogether, these results identify molecules involved in a novel functional cross-talk occurring between CD38$^+$CD49d$^+$ CLL and microenvironmental cells, this interplay eventually affecting survival and recirculation of tumor cells via the CD49d/VCAM-1 pair.

P129

MOLECULAR AND CLINICAL FEATURES OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA WITH OR WITHOUT STEREOTYPED B-CELL RECEPTORS: AN ITALIAN MULTICENTRIC EXPERIENCE

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Stereotyped B-cell receptors (BCR) occurred more frequently in chronic lymphocytic leukemia (CLL) with unmutated (UM) than mutated (M) immunoglobulin heavy chain variable (IGHV) genes. In 1398 CLL patients, we investigated 1426 IGHV rearrangements using a heavy chain complementary-determining region-3 (HCDR3)-driven clustering by means of the ClustalX(1.83) program, and correlated the molecular findings with time-to-treatment (TTT) and the presence of known prognosticators (Rai staging, CD38, ZAP70 and CD49d expression, karyotype abnormalities evaluated by interphase FISH). We identified 71 clusters with stereotyped BCR (325 cases, 22.8%), 32 with at least 3 cases/cluster ("confirmed" clusters) overall comprising 247 CLL (17.3%, UM/M=166/81). Sixteen out of 32 clusters were common to other CLL datasets, while 16 were novel clusters. Among these novel clusters, 11 comprised cases with M IGHV rearrangements, 8/11 involving the IGHV3 gene family; specific "cluster-biased" stereotyped amino acid (AA) changes were found also in IGHV sequences of these clusters. Cases belonging to the IGHV3-21/IGLV3-21 cluster had TTT similar to UM CLL and shorter than the whole series of M CLL (p<0.001) or of M CLL expressing IGHV3-21 but not in cluster (p=0.016). Consistently, CLL belonging to this cluster more frequently expressed unfavourable prognosticators than IGHV3-21 CLL not included in cluster (p=0.002). Moreover, cases belonging to the IGHV1-2/1-3/1-18/1-46/7-4-1/IGKV1-39 cluster had poorer prognosis than UM/M cases (p<0.001), or UM/M cases expressing the same IGHV genes but not in clusters (p=0.009). Unfavourable prognosticators were more frequent in CLL of this cluster than in their counterparts not in clusters (p=0.009). CLL expressing the IGHV3-23 gene virtually never belonged to clusters (4/134 cases in clusters). Alignment of IGHV sequences of IGHV3-23 CLL revealed a high degree of conservation in the context of the 13 AA positions involved in superantigen binding by IGHV3 family genes. TTT in IGHV3-23 M CLL was shorter than in M CLL (p=0.019), or in M CLL expressing IGHV3 family genes (p=0.021). Accordingly, multivariate Cox proportional hazard analyses selected IGHV3-23 usage (p=0.013), along with Rai staging (p=0.0025) and interphase FISH (p=0.0025) as independent prognosticators for M CLL. Altogether, novel molecular and clinical features were provided for CLL expressing or not stereotyped BCR.

P130

TP53 MUTATIONS AND DEL17P13 PREDICT SIMILAR OUTCOME AND CHEMOREFRACTORINESS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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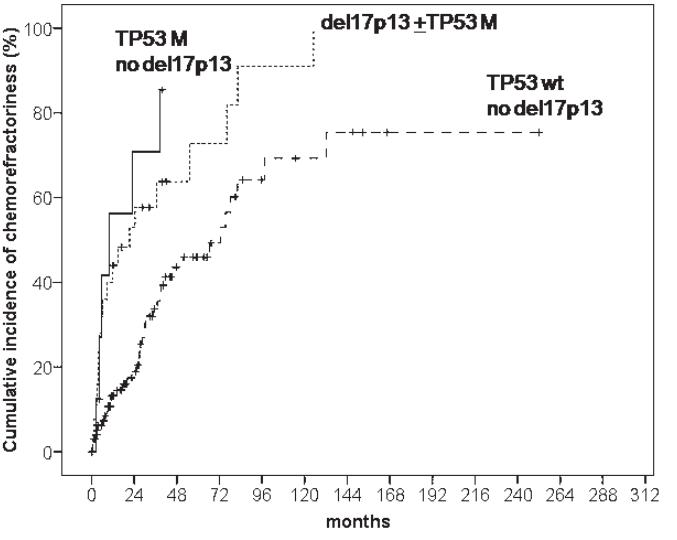
Chronic lymphocytic leukemia (CLL) harboring del17p13 displays a dismal prognosis and a high risk of chemorefractoriness. The tumor suppressor TP53 is located on chromosome band 17p13 and loss of TP53 is thought to be responsible for the poor prognosis of del17p13 CLL. This study aimed at verifying whether TP53 inactivation through mutation harbors the same prognostic role as del17p13. The study was based on a consecutive series of 308 CLL provided with a large and homogeneous dataset of biological and clinical variables. TP53 mutation status was assessed on PBMC collected at CLL diagnosis by direct sequencing of TP53 exons 2 to 10. Thirty-two TP53 mutations were observed in 31/308 (10.0%) CLL. Mutations were missense in 27/32 (84.3%) cases, short deletions in 3/32 (9.3%) and insertion and splicing site mutations in 1/32 (3.1%) cases each. TP53 mutations affected exon 4 in 2/32 (6.2%) cases, exon 5 in 6/32 (18.7%), exon 6 in 5/32 (15.6%), exon 7 in 9/32 (28.1%), and exon 8 in 10/32 (31.2%). No mutations were found in exons 2, 3, 9 or 10. Mutations targeted the TP53 DNA-binding domain in 29/32 (90.6%) cases. Functional codons targeted by mutations were TP53 DNA-binding codons (8/32, 25.5%) and zinc-ligand codons (3/32, 9.3%) by http://www-p53.iarc.fr/StructureAnalysis.html. All mutations predicted a reduction of trans-activation activity of the mutated p53 protein (median residual activity: 8.9% by http://p53.free.fr/Database/p53_recomendations.html). FISH karyotype was available in 297/308 cases. Overall, 44/297 (14.8%) CLL harbored TP53 inactivation through deletion and/or mutation: 18/297 (6.1%) CLL were TP53-mutated/del17p13, 16/297 (5.4%) were TP53-wild type (wt)/del17p13, and 10/297 (3.4%) were TP53-mutated/no del17p13. Median residual TP53 trans-activation activity was similar between the TP53-mutated/no del17p13 and the TP53-mutated/del17p13 subgroups (17.4% *vs.* 10.3% respectively; p=0.101). Univariate log-rank analysis identified TP53 mutations as a risk factor of short treatment free survival (TFS) (TP53-mutated: 18.3 months *vs.* TP53-wt: 80.4 months; p<0.001), overall survival (OS) (TP53-mutated: 79.6 months *vs.* TP53-wt: not reached; p<0.001), time to chemorefractoriness (TP53-mutated: 6.3 months *vs.* TP53-wt: 72.7 months; p<0.001), time to alkylator refractoriness (TP53-mutated: 6.0 months *vs.* TP53-wt: 66.5 months; p<0.001), and time to fludarabine refractoriness (TP53-mutated: 11.9 months *vs.* TP53-wt: 82.1 months; p<0.001). TFS, OS and time to chemorefractoriness (Figure 1) did not differ between CLL harboring TP53 mutations in the absence of del17p13 versus CLL harboring del17p13 (TFS: TP53-mutated/no del17p13: 17.2 months, del17p13/TP53-mutated or TP53-wt: 12.1 months, p=0.730; OS: TP53-mutated/no del17p13: 79.6 months, del17p13/TP53-mutated or TP53-wt: 104.2 months, p=0.937; time to chemorefractoriness: TP53-mutated/no del17p13: 9.8 months, del17p13/TP53-mutated or TP53-wt: 21.4 months, p=.483).



	Events/N	Median
TP53 M/no del17p13	6/8	9.8 mo
del17p13 ± TP53 M	19/25	21.4 mo
TP53 wt/no del17p13	38/101	72.7 mo

Figure 1.

Multivariate analysis selected TP53 mutation as an independent predictor of OS (HR:3.16; p=0.004) and chemorefractoriness (HR:6.68; p<0.001) after adjustment for age, sex, Binet stage, B2M, LDH, Hb, lymphocytes, platelets, IGHV gene homology, CD38 and ZAP70 expression, FISH karyotype, lines of treatment and exposure to fludarabine. These results document that mutation in the absence of del17p13 is the sole mechanism of TP53 inactivation in a fraction of CLL. TP53 mutations carry the same prognostic relevance as del17p13 in terms of CLL progression, survival and risk of chemorefractoriness. Because of the practical implications for choice of therapy, screening for TP53 mutations, in addition to del17p13 assessment, should be included in the initial prognostic assessment of CLL.

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SHORT TELOMERE LENGTH IS AN INDEPENDENT PREDICTOR OF SURVIVAL, PROGRESSION, RICHTER'S SYNDROME TRANSFORMATION AND RECURRENT INFECTIONS: AN ANALYSIS ON 421 CLL PATIENTS INCLUDING BLINDED VALIDATION ON 230 CASES

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Background. In chronic lymphocytic leukemia (CLL) short telomeres have been associated to poor outcome. However validation on a large series is required to verify if TL is worth entering clinical practice. Aims. i) to definitely validate TL as an independent prognostic factor for time to first treatment (TTFT) and overall survival (OS) in a large CLL series (including a large blinded sample); ii) to assess TL impact on other relevant CLL endpoints, including Richter's syndrome (RS) transformation and infection risk. Methods. We used two consecutive series of 421 CLL (historical series n=191 from University of Turin; blinded validation series n=230 from Avogadro University). TL was assessed on PBMC collected at diagnosis by Southern blotting. *Results.* ROC analysis identified a cut-off point of 4650bp. Median TL was 6000bp (25th-75th:4614-7487bp). TL<4650bp was observed in 100/387 (25.8%) CLL. TL<4650bp associated with advanced Binet stage (p=0.003), IGHV-homology>98% (p<0.001), del11q22-q23 (p=0.009), CD38>30% (p=0.018). Univariate log-rank analysis (UA) identified TL<4650bp as a risk factor (RF) of short TTFT (19.2 months *vs.* 85.2 months; p<0.001), along with Binet B-C, IGHV-homology >98%, CD38>30%, ZAP70>20%, del11q22-q23, del17p13, +12, and absence of del13q14 (p<0.001 in all instances). Multivariate analysis selected TL<4650bp as an independent TTFT predictor (HR:2.04; p=0.002). UA identified TL<4650bp as a RF of short OS (82.2 *vs.* 269.3 months; $p=3.5\times10^{-9}$), along with age>65y, Binet B-C, IGHV-homology >98%, and del17p13 (p<0.001 in all instances). Multivariate analysis selected TL<4650bp as an independent OS predictor (HR:3.15; p=<0.001). TL<4650bp consistently identified a subset of patients with short TFS and OS, despite harboring favorable predictors as documented by bivariate log-rank test . Indeed, TL<4650bp segregated a CLL group displaying short TTFT despite being characterized by Binet A (1.7×10^{-8}), IGHV-homology<98% ($p=1.6\times10^{-10}$), CD38<30% ($p=1.4\times10^{-8}$), ZAP70<20 ($p=3.3\times10^{-11}$), and normal FISH or del13q14 only ($p=4.1\times10^{-11}$). Also, TL<4650bp segregated a CLL group displaying short OS despite having age<65y ($p=1.4\times10^{-7}$), Binet A (p=0.001), IGHV-homology <98% ($p=1.4\times10^{-6}$), and del17p13 absence ($p=6.2\times10^{-5}$). Results were superimposable in the historical and blinded validation series. The validation series (n=230) allowed the investigation of additional outcomes, namely RS transformation and infection risk. UA identified TL<4650bp as a RF for RS (5-year risk: TL<4650bp 35.8% *vs.* TL>4650bp 6.3%; p=0.001. Multivariate analysis selected TL<4650 (HR:4.1, p=0.007) (Figure 1B) and lymphadenopathy >3cm (HR: 7.4, $p=2.1\times10^{-4}$) as independent predictors of RS. UA identified TL<4650bp as a RF of short time to recurrent infections (TL<4650bp 3.0 months *vs.* TL>4650bp 26.3 months; $p=1.9\times10^{-4}$). Multivariate analysis identified TL<4650bp (HR:6.35; p=0.002) and IGHV-homology >98% (HR: 3.51; p=0.007) as independent predictors of recurrent infections. Conclusions. This is the largest TL analysis so far in CLL and is provided with an independent and blinded validation cohort. The implications of our results are multifold. First, short TL is validated as an independent OS and TFS predictor. Second, short TL identifies a CLL subgroup with rapid disease progression and short survival despite the presence of favorable predictors. Third, short TL independently predicts RS and recurrent infections. Because short TL are the sole independent predictor of all CLL outcomes, we advise its more widespread clinical use.

P132

BCL3 TRANSLOCATION IS A FREQENT AND SELECTIVE EVENT IN CHRONIC LYMPHOCYTIC LEUKEMIA CARRYING +12

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BCL3 encodes a co-activator of the nuclear factor kappaB (NF-kB) 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. Though BCL3 translocation has been described in CLL, a comprehensive study aimed at defining the prevalence of BCL3 translocation in this setting is lacking. In this study we analysed the prevalence of BCL3 translocation in a consecutive series of 202 CLL provided with a dataset of biological and clinical characteristics at diagnosis. BCL3 translocation was investigated by FISH analysis using a break apart dual colour probe (DAKO, Cambs, UK). IGH translocation was investigated by IGH break apart dual colour probe (Vysis/Abbott, Downers Grove, IL, USA). Biological and clinical characteristics at diagnosis of the CLL series (n=202) were representative of the disease. Among biological features, IGHV homology >98% occurred in 70/200 (35.0%) cases, CD38 >30% in 59/202 (29.2%), ZAP70 >20% in 55/179 (30.7%), CD49d >30% in 71/184 (38.6%), del13q14 in 106/202 (52.2%), +12 in 46/202 (22.8%), del11q22-q23 in 14/202 (6.9%), del17p23 in 20/202 (9.9%), normal FISH in 51/202 (25.2%), TP53 mutation in 18/202 (8.9%). Among clinical features, median age was 69 years, 114/202 (56.4%) patients were men, 155/202 (76.7%) were in Binet stage A, 26/202 (12.9%) in Binet stage B, and 21/202 (10.4%) in Binet stage C. BCL3 was translocated in 4/201 (1.9%) CLL. IGH was the partner gene in all (4/4) CLL carrying BCL3 translocation. Among FISH categories at CLL diagnosis, BCL3 translocation was selectively associated with +12. Indeed, BCL3 was translocated in 4/46 (8.9%) CLL harbouring +12 *vs.* 0/156 CLL devoid of +12 (p=0.002). When considering CLL carrying +12, BCL3 translocation was restricted to CLL cases characterized by +12 as the sole FISH abnormality (4/30, 13.3%). Conversely, BCL3 translocation was absent among CLL cases carrying +12 and additional FISH abnormalities (0/16). Among CLL with BCL3 translocation, CD49d >30% was observed in 4/4 cases, IGHV homology >98% in 3/4, CD38>30% in 3/4, ZAP70 >20% in 2/4, TP53 mutations in 0/4, Binet A stage in 3/4. Concerning IGHV usage, IGHV4-34 was utilized by two cases, and IGHV3-48 and IGHV3-49 were utilized by one case each. The CLL series (n=202) was also investigated for IGH translocation by FISH analysis using IGH break apart dual colour probe. IGH translocations not involving BCL3 were observed in 11/202 (5.5%) CLL. The partner gene was BCL2 in 4 cases. The remaining 7 cases were negative for translocations involving BCL6, c-MYC, and MALT1. At variance with BCL3 translocation, IGH translocations not involving BCL3 affected both +12 positive (4/46, 8.7%) and +12 negative (7/156, 4.5%) CLL. The implication of our results are twofold. First, BCL3 translocation is a relatively frequent event in CLL carrying +12 as the sole FISH abnormality. Second, at variance with other IGH translocations, BCL3 translocation selectively clusters with CLL carrying +12. These observations prompt studies aimed at verifying the prognostic relevance of BCL3 translocation in CLL carrying +12.

P133

IGHV MUTATION STATUS PER SE IS THE STRONGEST PREDICTOR OF TIME TO FIRST TREATMENT (TTT) IN STAGE A AND B CHRONIC LYMPHOCYTIC LEUKEMIA, IRRESPECTIVE OF IGHV FAMILY AND GENE USAGE

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We assessed the impact of biological features on TTT, a validated indicator of disease progression, in a cohort of 286 consecutive untreated pts with stage A or B CLL. Pts characteristics: median age 58 yrs

(range 27-81), M/F: 178/108; stage A 250 pts (87%), B 36 (13%); median follow up: 39 mos (range 7-222). Biologic characteristics: ZAP70 expression (flow cytometry; cut off: ≥20% pos.CLL cells): 77 pts (27%); IgHV unmutated (UM; cut-off value for homology to germline: ≥98%; IMGT database): 118 pts (41%); FISH hierarchical classification: del13q: 42%, +12: 11%, del11q: 8%, del 17p: 5.5%, negative FISH panel: 32%. A significant association was detected between UM-IgHV and unfavourable FISH (del11q/del17p) and ZAP70+ (p<0.001); however, discordance between ZAP70 and IgHV mutational status was observed in 30% of pts. Male pts had higher frequency of UM IgHV and unfavourable FISH, while no difference was observed in different age subgroups. 132 pts (46%) received chemotherapy because of disease progression. Median TTT was 52 mos for the whole cohort of pts (Kaplan-Meier). IgHV mutation status was the strongest predictor of TTT in a Cox multivariate regression model (IgHV: p<0.001; unfavorable FISH: p=0.03; ZAP70: p=0.19), and we focused on this biologic feature. Pts were categorized according to the degree of homology to germline: 100%; 98-99.9%; 97-97.9%; 96-96,9% and <96%. Pts with 97-97.9% homology (17 pts, 6%) did not experience a different TTT as compared with <97% pts (53% of cases): median TTT 94 mos *vs.* 97 mos; conversely, median TTT was shorter in UM-pts (32 mos, Gehan-Wilcoxon test p=0.07). IgHV usage was non-random, with HV3, HV1 and HV4 families being expressed at the highest frequencies (51%, 26% and 19%, respectively). The most frequently rearranged IgHV genes were V1-69 (35 pts, UM 29), V3-30 (30 pts), V4-34 (26 pts), and V3-23 (25 pts); 7 pts had V3-21 (UM 4). IgHD3 (36% of 277 pts) and IgHJ4 (45% of 280 pts) were the most common D and J genes. No significant differences were observed in the distribution of HV usage according to gender and age. TTT was not significantly associated with a specific IgHV family. IgHV 1-69 pts received treatment more frequently and had a shorter TTT than IgHV non 1-69 pts (p=0.009), but these differences were not longer observed when UM pts only were included. In our cohort of CLL pts, IgHV mutation status seems to be the most important prognostic factor for progression and need of therapy.

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CD49D EXPRESSION IS AN INDEPENDENT PREDICTOR OF PROGRESSIVE DISEASE IN BINET A CHRONIC LYMPHOCYTIC LEUKEMIA

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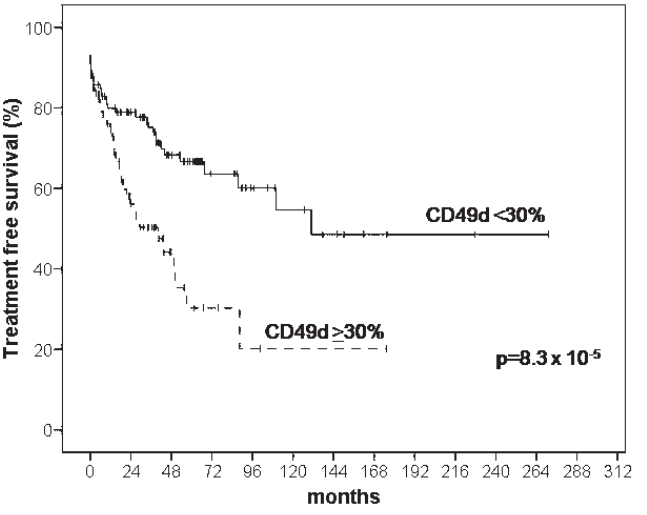
Chronic lymphocytic leukemia (CLL) is a markedly heterogeneous disease. This notion is best exemplified by the variability in time to progression and in survival of Binet A CLL. The identification of prognostic subgroups within Binet A CLL is currently a major challenge. CD49d represents a novel CLL prognosticator, whose value in Binet A patients is unknown. This study aimed at verifying whether CD49d expression may further refine the prognostic stratification of Binet A CLL. The study was based on a consecutive series of 140 Binet A CLL representative of this disease stage and provided with a large and homogeneous dataset of biological and clinical variables. CD49d expression was assessed on PBMC collected at CLL diagnosis by three-color flow cytometry. A cut-off point of 30% was utilized to define positivity. At diagnosis, CD49d>30% was observed in 54/140 (38.6%) Binet A CLL. CD49d>30% associated with markers of proliferating CLL, namely CD38>30% (p<0.001), short telomere (p=0.021), high LDH (p=0.007) and beta-2-microglobulin (p=0.020). CD49d expression was not associated (p>0.05 in all cases) with IGHV homology, del11q22-q23 or del17p13, number of FISH lesions,TP53 inactivation by deletion and/or mutation, or ZAP70 expression. Also, CD49d expression was not associated (p>0.05 in all cases) with clinical markers of tumor burden. Univariate log-rank analysis identified CD49d>30% as a risk factor of progressive disease, in terms of time to lymphocyte doubling (CD49d>30%: 28.2 months *vs.* CD49d<30%: 53.0 months; p=0.009), time to progression (CD49d>30%: 38.0 months *vs.* CD49d<30%: 66.7 months; p<0.001), and treatment free survival (TFS) (CD49d>30%: 50.2 months *vs.* CD49d<30%: not reached; p<0.001) (Figure 1). Multivariate analysis selected CD49d>30% as an independent TFS predictor after adjustment for biological (HR 2.39, p=0.019), clinical (HR 4.34, p<0.001), and both biological and clinical variables analysed together (HR 2.59, p=.032). Within Binet A subgroups harboring favorable predictors, CD49d>30% consistently identified a subset of patients with short TFS, as documented by bivariate log-rank test. Indeed, among patients harboring favorable biological predictors, CD49d>30% segregated a group of CLL displaying short TFS despite being characterized by IGHV homology <98% (p=0.007), number of FISH lesions <1 (p<0.001), normal FISH or del13q14 only (p=0.005), wild type TP53 (p<0.001), telomere length >4250 bp (p<0.001), CD38<30% (p=.005), or ZAP70<20% (p=0.003). Also, among patients harboring favorable clinical predictors, CD49d>30% identified a group of CLL displaying short TFS despite being characterized by Rai 0 (p=0.001), absence of splenomegaly (p<0.001), lymphocytes <20×10^9 (p=0.002), Hb >13 g/dL (p<0.001), platelets >150×10^9/L (p<0.001), BM lymphocytes <50% (p=0.009), nondiffuse BM pattern (p=0.001), beta-2-microglobulin <2.5 mg/L (p=0.003), and LDH <1×ULN (p=0.007). Overall, the results are consistent with CD49d>30% being a marker of disease proliferation and dissemination in Binet A CLL. Our observations, along with the easiness of CD49d assessment, indicate CD49d>30% as a new marker for the initial prognostic assessment of Binet A CLL. In particular, CD49d>30% may be useful for the identification of a subgroup of Binet A CLL that display rapid disease progression and need of treatment, despite being characterized at diagnosis by favorable predictors.



	Events/N	Median	5-year risk
CD49d ≥30%	23/54	50.2 mo (38.8-61.7 mo)	38.5% (18.6-54.8%)
CD49d <30%	17/86	Not reached	79.0% (69.1-88.9%)

Figure 1.

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MONITORING FOR CMV AND EBV INFECTION IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS RECEIVING FLUDARABINE-CYCLOPHOSPHAMIDE (I.V. FC) COMBINATION AND ALEMTUZUMAB (AL) AS CONSOLIDATION OF RESPONSE

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We monitored for CMV and EBV infection 67 CLL pts receiving standard dose i.v. FC combination (median number of FC courses 5, range 3-6; 53 previously untreated, 14 after single agent therapy; median age 54 yrs, range 29-65;). Seventeen pts with residual disease (4-color flow cytometry) received i.v./s.c. AL as consolidation (30-60 mg/wk for 3-10 wks, starting 3-4 mos after FC completion). CMV reactivation was monitored by measuring pp65 antigenemia on peripheral blood leukocytes

(PBL) or by detection of CMV-DNA in whole blood by a quantitative PCR assay. EBV was monitored by measuring EBV-DNA copies/10^5peripheral blood mononuclear cells (PBMC) by real-time PCR assay. All pts received oral prophylaxis (acyclovir or valacyclovir) for HSV infections; corticosteroids were not administered. Pts were monitored every 2 wks during FC, monthly for 3 mos after FC, and weekly during consolidation. CMV and EBV serologies were consistent with prior infection in 90% and 96% of pts, respectively. During FC therapy, CMV infection was never detected, while asymptomatic and transient increase in EBV viral load (median peak level 108 DNA copies/10^5 PBMC; range 15-650) was observed in 17 pts (25%). CD4 T-cell counts decreased from median baseline value of 490/microL (range 142-1965) to a median post-treatment value of 167/μL (range: 71-680) ($p<0.05$). During AL consolidation, CMV-DNAemia was detected in 9 pts (52%) with peak levels ranging from 3 to 70 pp65-positive cells/2×10^5 PBL or from 3500 to 7900 CMV-DNA copies/mL whole blood. Ganciclovir was administered as preemptive therapy in 5 asymtomatic pts and for symptomatic infection in 4 (fatigue, fever, cough, arthralgias; CMV load in these pts: 70 antigenemia positive cells/2×10^5 PBL, CMV-DNAemia 3500, 4600, 7900 copies/mL whole blood, respectively). Eight pts (47%) showed an increase in EBV-DNA load with peak levels ranging from 95 to 6700 copies/10^5 PBMC (median peak level: 624); EBV-DNA load of 6700 copies/10^5 PBMC was associated to EBV-related disease progression in 1 pt. Detection of CMV or EBV in blood was not related to the disease stage or to AL cumulative dose. All CMV and EBV-positive pts recovered from infectious episodes. These results indicate that CMV and EBV infection is uncommon in pts receiving FC as first or second-line treatment. In contrast, close surveillance is mandatory in the setting of consolidation with AL and should include serial quantification of EBV-DNA load in PBMC as well as CMV load.

P136

BORTEZOMIB INDUCES APOPTOSIS IN BOTH RESTING AND CD40-ACTIVATED CLL B CELLS

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Background. Proteasome inhibition by its specific inhibitor, bortezomib (VELCADE) has recently become an innovative anti-tumor strategy, approved for the clinical use in patients with resistant or relapsed multiple myeloma. The emerging role of bortezomib in the treatment of indolent lymphomas has been shown in recent clinical studies. Bortezomib proved to be active also against chronic lymphocytic leukemia (CLL) B cells, inducing their apoptosis *in vitro*. However, experiences from clinical trials evaluating bortezomib as single agent in small lymphocytic lymphoma and B-CLL patients were unexpectedly disappointing. It was hypothesized that *in vivo* stimuli may confer resistance to apoptosis in CLL B cells. In this study, we induced CLL B cells to CD40-mediated activation prior to bortezomib exposure, in order to assess whether activating stimuli may play a role in resistance to bortezomib *in vivo*. Indeed, CD40-mediated activation of B cells is known to result in upregulation of anti-apoptotic molecules, which in turn confers a survival advantage to stimulated B cells. *Patients and Methods.* Thus far, 8 untreated patients with B-CLL in various Rai stages have been studied. Bortezomib was used at a concentration of 2.5 nM, which was chosen following a series of time-course experiments with different concentrations (1.0, 2.5, 5.0, and 10 nM). Resting CLL B cells were incubated with bortezomib (2.5 nM) overnight and apoptosis was assessed by annexin-V binding and propidium iodide staining using flow cytometry. For activation of CLL B cells, leukemic lymphocytes were incubated overnight with soluble human recombinant CD40L (100 ng/mL). Upregulation of CD86, CD95, and CD69 was used to document activation. Then, activated CLL B cells were incubated with bortezomib for 18-22 h prior to apoptosis assessment as described above. *Results.* Bortezomib was able to induce apoptosis in both resting and CD40-activated CLL B cells with comparable efficacy. After overnight exposure to bortezomib, apoptosis rates were shown to approach nearly 100%, regardless activation induction. Thus, there were no significant differences in susceptibility to apoptosis between resting and CD40-activated CLL B cells. Even the only ZAP-70-positive case recruited so far was shown to rapidly undergo extensive apoptosis. *Conclusions.* Bortezomib was effective in inducing apoptosis *in vitro* even in stimulated CLL B cells. Further studies are needed to identify the reasons for *in vivo* resistence to bortezomib in B-CLL patients.

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IGHV1-69-EXPRESSING CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): CHARACTERIZATION OF 36 PATIENTS

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IGHV1-69–using CLL has been related to a restricted IGH gene features and poor prognosis. We analysed characteristics and outcome of 36 IGHV1-69 pts from a cohort of 295 untreated CLL pts (12.5%). Staging was as follows: 29 stage A, 6 stage B, 1 stage C; stage distribution was not different as compared with VH non 1-69 pts. M/F ratio was 3.5 (1.6 for VH non 1-69 pts); median age was not different (58 yrs). IGH: IgM in 35/36 pts; IgL: kappa 70% and lambda 30%. Thirty pts (83%) were unmutated (UM) with 100% germ-line homology; VH1-69 was the most frequently used gene in the UM group (24%) (sequences were aligned to IMGT database). In contrast, among VH1-69 pts 36% were UM ($p<0.001$). ZAP70 expression and unfavourable FISH (del11q/del17p) were more frequent in the VH1-69 group, but the difference was not significant. We were able to determine both the IGHD and IGHJ gene usage in 35/36 pts: the most frequent IGHD genes were DH2-2, DH3-3 and DH3-10; JH6 and JH4 accounted for 83% of cases. The combination of IGHD3 and IGHJ6 (28%) is of interest, due to its previously reported association with VH1-69 gene. HCDR3 aa length (assessed in 35/36 pts) varied from 14 to 27; median aa length was considerably shorter in the M cases (15 *vs.* 22 aa). Sequences were clustered based on particular HCDR3 aa motifs to identify cases with homologous HCDR3 (>60% aa homology) and were compared with the stereotyped HCDR3 subsets previously identified (Murray F *et al*, 2008). Eighteen pts (6 M, 12 UM) carried different aa sequences and did not belong to any of the reported subsets. Conversely, 17 UM pts carried restricted HCDR3s and could be grouped into 7 different subsets: 7 pts in subset #7; 3 pts in subset #9; 2 pts in subset #3; 2 pts in subset #69; 1 pt each in subset #6, #50, #75. The influence of IGHV1-69 on outcome was evaluated including only stage A and B pts: VH1-69 pts received treatment more frequently (69% *vs.* 45%) and had a significantly shorter time to treatment (TTT as indicator of disease progression), than VH non 1-69 pts (29 mos *vs.* 58 mos; Gehan-Wilcoxon test $p=0.009$); however, these differences were not longer observed when UM pts only were included (median TTT 25 mos *vs.* 33 mos; $p=0.6$). Our results suggest that: 1) the expression of IGHV1-69 gene is not predictive of poor prognosis, as the outcome in these pts may be more specifically related to the unmutated status; 2) biased IGHV-D-J gene usage may support the hypothesis of specific antigen involvement in CLL.

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HIGH CD54 ADHESION MOLECULE EXPRESSION PREDICTS A SHORT PROGRESSION FREE SURVIVAL IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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CD54/intercellular adhesion molecule-1 (ICAM-1) is generallly found at lower levels in B-cell chronic lymphocytic leukemia (B-CLL) and its higher expression has been already associated with marked organomegaly and poor prognosis (Hjalmar, 2002). Furthermore, it has been clearly demonstrated that ZAP-70$^+$ CLL shows a rapid disease progression and an inferior overall survival (Crespo, 2003; Del Principe, 2006). The primary aims of our research were: 1) to correlate CD54 with other recent biologic prognostic factors, 2) to determine progression-free survival (PFS) upon CD54 expression; 3) whether CD54 could predict varied outcome within ZAP-70$^+$ and ZAP-70 negative subgroups; and finally, 4) to test the independent value of CD54 as prognosticator. Therefore, we investigated 202 pts, median age 65 years (range 37-87), 105 males and 97 females. With regard to modified Rai stages, 64 pts had a low stage, 132 an intermediate stage and 6 a high stage. CD54 was determined by multicolor flow cytometry fixing a cut-off value of 30%. CD54$^+$ B-CLL pts were 131/202 (65%). CD54>30% was associated with an intermediate/high Rai stage (p=0.008), with marked lymphadenopathy and/or splenomegaly (p=0.00001) and with beta-2 microglobulin >2.2 mg/dL (p=0.0006). Also, high CD54 and IgVH unmutated status (<2%) were correlated (25/29; p=0.001). Furthermore, significant associations were found either between higher CD54 and higher ZAP-70 (59/79; p=0.01) or between higher CD54 and higher soluble CD23 (sCD23) levels (48/59; p=0.002). A significant shorter PFS was observed in CD54$^+$ pts (22% *vs.* 72% at 14 years; p<0.00001, Figure 1) as well as in ZAP-70$^+$ pts (15% *vs.* 63% at 10 years; p<0.00001). To further explore the prognostic impact of CD54, we investigated its expression within ZAP-70$^+$ (79 pts) and ZAP-70 negative (123 pts) subsets. As a matter of fact, CD54$^+$ pts showed a shorter PFS both within the ZAP-70$^+$ subset (13% *vs.* 22% at 10 years; p=0.03) and within the ZAP-70 negative subset (42% *vs.* 91% at 14 years, p=0.0001). In multivariate analysis of PFS both ZAP-70 (p=0.0001) and CD54 (p=0.004) resulted to be independent prognostic factors. Therefore, CD54, determined by flow cytometry, could be considered as a promising prognostic parameter in B-CLL. Moreover, since the ZAP-70 negative subgroup consists of a large and a heterogeneous population presenting variable outcome, CD54 should be used to identify B-CLL progressive pts, particularly those with important organomegaly.

Chronic Lymphocytic Leukemia (II)

P139

MOLECULAR AND TRANSCRIPTIONAL CHARACTERIZATION OF 17P LOSS IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Distinct genetic abnormalities such as TP53 deletion at 17p13.1 have been identified as having adverse prognostic relevance in B-cell chronic lymphocytic leukemia (B-CLL), and conventional cytogenetic studies have shown that TP53 deletion in B-CLL is mainly associated with the loss of 17p due to complex chromosomal rearrangements. We used an integrative genomic approach to investigate the significance of 17p loss in a subset of B-CLLs carrying a TP53 monoallelic deletion detected by Fluorescence in-situ hybridization (FISH). A panel of 71 untreated Binet A B-CLLs (18 carrying 17p-) was characterized for the most recurrent genomic aberrations and for the major prognostic markers. The genomic profile of chromosome 17p was investigated with GeneChip Human Mapping 50K Xba arrays in 12/18 17p- B-CLLs. Inferred copy numbers were derived from a Hidden Markov Model (HMM) based algorithm implemented in CNAT 4.0.1 software (Affymetrix). FISH probes covering a region of approximately 6 Mb in 17p11.2-p12 was selected to validate the array results. The transcriptional profiles of the 60 B-CLLs (7 carrying 17p-) have been generated on Affymetrix GeneChip U133A arrays. The identified transcriptional fingerprints of the 17p- cases was validated on an independent dataset of 100 B-CLL cases (Haslinger *et al*., 2004) using a Multi-class Prediction Analysis. Polymerase chain reaction was used to define the mutational status of the TP53. Genome-wide DNA analysis of TP53-deleted samples showed 17p loss in 11/12 cases, with breakpoints scattered along the 17p11.2 region. FISH analysis confirmed these findings and revealed 17p loss in a small fraction of leukemic cells in the remaining TP53-deleted case. In addition, FISH indicated 17p loss in the 6/18 cases not investigated by SNP. Mutations in exons 2 to 11 of the remaining TP53 allele were found in 9/12 17p- B-CLLs. Gene expression profiling of 60 B-CLLs, identified 40 differentially expressed genes in 17p- versus 17p normal samples, 35 of which were down-regulated in 17p- tumors: the majority (30/35) of these transcripts, including putative tumor suppressor genes (GABARAP, GPS2 and OVCA1) mapped to 17p, indicating a remarkable gene dosage effect. Our data provide evidence that 17p loss may play an additional pathogenetic role in B-CLL, and suggest that the concomitant loss of multiple tumor suppressor genes could be responsible for the highly adverse prognostic relevance associated with TP53 loss.

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ANALYSIS OF B-CELL RECEPTOR SIGNAL TRANSDUCTION IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA BY PHOSPHOSPECIFIC FLOW-CYTOMETRY

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B-cell chronic lymphocytic leukemia (B-CLL) is a clonal lymphoproliferative disease characterized by the expansion of mature CD5+ B-cells. Recent studies indicated that variability in clinical outcomes observed in CLL patients is related to differences in the ability to transduce B-cell receptor (BCR)-mediate signals. The BCR signal transduction routes include, in the initial phases, Syk tyrosin kinase and BCR prolonged stimulation has been associated to activation of kinases includ-

ing mitogen activated protein kinases (MAPK). In this study we used phosphospecific flow cytometry to study single-cell signaling triggered by BCR in peripheral blood B-cells from 21 B-CLL patients. The following proteins were analyzed: Syk, MAPK (Erk, p38, JNK) and NF-kappaB, either in basal condition or after BCR cross-linking achieved by treatment with anti-IgM F(ab')2. Most cases showed constitutive phosphorylation of Syk and NF-kappaB (86% and 93%, respectively). Erk was constitutively phosphorylated in 81% cases, JNK in 5%, and p38 in 0% (Table 1). The BCR cross-linking increased the constitutive phosphorylation of Syk in 50% cases, of Erk in 27%, and of NF-kappaB in 21%. No cases showed p38 or JNK increased phosphorylation (Table 1). Recent works shows that efficient activation of BCR signaling depends on inactivation of phosphatase proteins (PTPs), and that endogenously generated H_2O_2 is an important transiently PTPs inhibitor. Therefore, we cross-linked the BCR in the presence of H_2O_2 . In this condition we observed an increase of phosphorylation of all proteins with respect to treatment with anti-IgM alone (Table 1). Furthermore, the treatment of B-CLL cells with H2O2 alone induced increased phosphorylation of signaling proteins with respect to the constitutive level (Syk in 21%, p38 in 56%, Erk in 38%, JNK in 9%, and NF-κB in 43% cases, Table 1). Conversely, PTP inhibition in normal B cells did not significantly modify the phosphorylation of these proteins. Taken together, these results revealed a clear-cut remodeling of BCR-mediated signaling in B-CLL cells with respect to normal B cells, thus confirming and extending previous results obtained by Western blot analysis. Furthermore, this study provides the first analysis of BCR signaling by phosphospecific flow cytometry in B-CLL cells and indicate that single-cells measurement of phosphoproteins in response to BCR engagement enables to define cell network phenotypes in B-CLL by multidimensional molecular profiles of signaling. *Supported by Regione Veneto Ricerca Sanitaria Finalizzata and Fondazione G. Berlucchi per la Ricerca sul Cancro.*

Table 1.

	P-Syk	*P-p38*	*P-Erk1/2*	*P-JNK*	*P-NF-kappaB*
Unstimulated*	12/14 (86%)	0/23 (0%)	17/21 (81%)	1/21 (5%)	13/14 (93%)
anti-IgM $F(ab')_2$ °	7/14 (50%)	0/17 (0%)	4/15 (27%)	0/15 (0%)	3/14 (21%)
anti-IgM $F(ab')_2$ plus H_2O_2 °	10/14 (71%)	13/17 (76%)	12/15 (80%)	3/15 (20%)	6/14 (43%)
H_2O_2°	3/14 (21%)	13/23 (56%)	8/21 (38%)	2/21 (9%)	6/14 (43%)

**Positives were considered when ratio between mean fluorescence intensity (MFI) of unstimulated cells and MFI of isotypic control was ≥1.5. °Positives were considered when ratio between MFI of stimulated cells and MFI of unstimulated cells was ≥1.5.*

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A DISTINCTIVE TRANSCRIPTIONAL PROFILE CHARACTERIZES TRISOMY 12 IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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B-cell chronic lymphocytic leukemia (B-CLL) is a genetically heterogeneous disease with a variable clinical course. Trisomy 12, one of the most frequent abnormalities found in B-CLL, has been shown to be associated with a more aggressive clinical course. Gene expression profiling and genome-wide DNA analyses were performed in order to identify additional lesions and signalling pathways associated with trisomy 12. A panel of 80 Binet A untreated B-CLLs characterized for the most recurrent genomic aberrations and the major prognostic markers was investigated with Affymetrix GeneChip U133A expression arrays. Gene expression profiling identified a set of 140 genes as best classifier for the trisomy 12 B-CLLs (12/80 patients); 92 out of the 118 genes with significantly higher average expression in patients with trisomy 12 mapped on chromosome 12, mostly of which (86%) on 12q. The remaining upregulated genes and the 22 genes with significantly lower average expression had different chromosomal localizations. The identified transcriptional fingerprint was validated on an independent dataset of 100 B-CLLs (Haslinger *et al.*, 2004) using a Multi-class Prediction Analysis (global classification rate of 92.5%). A functional analysis of the deregulated genes revealed their involvement in different cellular functions such as transcription, mRNA and protein metabolism, cellular metabolism, DNA metabolism, cell-cycle and immune response. Genome-wide DNA profiling data were generated on GeneChip Human Mapping 250K Nsp arrays (Affymetrix) in a subset of 45 B-CLL patients. Conventional agglomerative hierarchical clustering analysis identified three consistent clusters, grouping: (I) all patients with chromosome 13 deletion (16/45, 36%); (II) all cases carrying trisomy 12 (9/45, 20%); and (III) either cases with 11 or 17 deletion as a sole abnormality or patients without any of the most frequent alterations. Other recurrent copy number variations (frequency > 6%) were identified, such as gains of 2p, 8q24, 14q32, 15q11, 17q21 and losses of 1q44, 4p, 6q12, 8p23, 9p23, 11p15, 14q11, 14q32 and 15q11: however, no significant association with any of the major genetic lesions was observed. Our data support the role of integrative genomics to provide insights into the characterization of molecular lesions in B-CLL.

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CLL TRANSFORMING TO RICHTER'S SYNDROME CARRY STEROTYPED HCDR3S AT VERY HIGH FREQUENCY (>50%) AND DISPLAY BIASED USAGE OF HOMOLOGOUS IGHV4-39 GENES

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Background. The prognostic value of specific IGHV gene usage is a well recognized feature of CLL. Usage of IGHV4-39 at CLL diagnosis appears to be an independent risk factor of transformation to Richter syndrome (RS) in multivariate analysis (Rossi, Br J Haematol 2008, in press). The potential relevance of specific IGHV gene usage in RS transformation may be further supported by the identification of stereotyped B-cell receptors in RS. Aim: To investigate HCDR3 clustering in CLL transformed to RS. *Methods.* The study was based on 80 CLL transformed to RS. A consecutive series of 571 CLL was utilized as internal control group. Analysis of IGHVDJ sequences was based on the International ImMunoGeneTics resource (IMGT®, http://imgt.cines.fr). Cluster analysis was performed with a blind approach using the multiple sequence alignment ClustalX (1.83) software. HCDR3s of CLL transformed to RS and HCDR3s of CLL belonging to the internal control group were aligned to HCDR3s of 2477 CLL from our collaborative multicentric database (Bomben, Blood 2007;110:909a) and from a previously reported database (Murray, Blood 2008;111:2083-2090). Cluster nomenclature was according to Stamatopoulos (Blood 2007;109:259-270) and Murray (Blood 2008;111:2083-2090). *Results.* Cluster analysis revealed that 45/80 (56.2%) CLL transformed to RS carried stereotyped HCDR3s. Prevalence of stereotyped HCDR3s was significantly higher in CLL transformed to RS than in CLL of the internal control group considering: i) all cases (RS: 45/80, 56.2% *vs.* CLL: 122/571, 21.3%; $p<0.001$); ii) IGHV unmutated cases (RS: 38/59, 64.4% *vs.* CLL: 70/198, 35.3%; $p<0.001$); and iii) IGHV mutated cases (RS: 7/21, 33.3% *vs.* CLL: 53/373, 14.2%; $p=0.014$). In addition, the prevalence of stereotyped HCDR3s in CLL transformed to RS was significantly higher than in

CLL from the two databases investigated (Murray, Blood 2008;111:2083-2090; Bomben, Blood 2007;110:909a) when the analysis was conducted: i) for all cases (p<0.001 and p<0.001, respectively); ii) for IGHV unmutated cases (p<0.001 and p<0.001, respectively); and iii) for IGHV mutated cases (p=0.02 and p=0.016, respectively). Analysis of IGHVDJ revealed that CLL transformed to RS displayed a biased usage of IGHV4-39 (9/80; 11.2%) when compared to the consecutive CLL series used as internal control group (9/571, 1.5%) (p<0.001). The biased usage of IGHV4-39 in CLL transformed to RS was also confirmed when CLL from the two databases were used for comparison (Murray database: IGHV4-39 *96/1967*, 4.8%, p=.009; Bomben database: IGHV4-39: *55/1426*, 3.8%, p=0.002). IGHV4-39 CLL transformed to RS entered a cluster in 9/9 cases (100%). According to the nomenclature by Stamatopoulos and Murray, seven cases belonged to subset 8, and 1 case to subsets 10 and 33 each. IGHV4-39 CLL transformed to RS entered a cluster more frequently than CLL from the databases (Murray database: *31/96*, 32.2%, p<0.001; Bomben database: IGHV4-39: *19/55*, 34.5%, p=0.001). *Conclusions.* The implications of these data are twofold. First, occurrence of stereotyped HCDR3s is a frequent feature of CLL that transform to RS, potentially suggesting an important contribution of antigen stimulation in driving CLL transformation to aggressive lymphoma. Second, these data document that IGHV4-39 preferentially associates with RS. The pathogenetic relevance of IGHV4-39 in RS transformation is further supported by evidence of stereotyped B-cell receptors in the overwhelming majority of RS cases utilizing IGHV4-39.

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REGIONE VENETO STUDY GROUP ON B-CLL: STANDARDIZATION OF ZAP-70 EVALUATION BY FLOW CYTOMETRY

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B-cell chronic lymphocytic leukemia (B-CLL) is the most frequent form of leukemia in developed countries. In the last years, major advances have been made in the identification of molecular and cellular markers that may predict disease progression especially in early stage of disease. Mutational status of IgVH genes, cytogenetic abnormalities, and immunophenotypic markers (such as CD38 and ZAP-70) are tightly associated with the prognosis of B-CLL. ZAP-70 is one of the most promising because of its strong correlation with IgVH mutational status. However, controversy is still present on what the best procedure to detect ZAP-70 status might be. As part of the Regione Veneto B-CLL Study Group Cytometry Working Party (composed by seven independent cytometry labs), we assessed the possibility to develop a common analysis for B-CLL, with special interest on ZAP-70 expression. At the end, consensus was reached on the sample preparation procedure, the immunophenotypic staining, and the data acquisition and analysis. Briefly, whole-blood cells were firstly stained for the surface antigens CD19 and CD5. After permeabilization, we stained cells with three different monoclonal antibodies (mAbs) against ZAP-70 (1E7.2, 2F3.2, SBZAP). Isotype matching immunoglobulins were used as negative control. Analysis was performed by three different methods, as follows. After immunological identification of B-CLL cells ($CD19^+/CD5^+$), we assessed the percentage of $ZAP\text{-}70^+$ B-CLL cells by comparison with an internal positive control (T cells: $CD5^+/CD19^-$), or with the isotypic control. Moreover, we analyzed the mean fluorescence intensity (MFI) for ZAP-70 in B-CLL cells in comparison with T-cells. From January 2008 on, we studied the ZAP-70 expression in 23 untreated B-CLL patients using this flow-cytometric standardized approach. Preliminary results show high discordance either when using the three different mAbs and when using the three different approaches for data analysis (Table 1). Moreover, evaluation of MFI appears to be more reproducible among different operators than estimation of the percentage of $ZAP\text{-}70^+$ cells. However, further analysis should be performed in order to investigate the actual correlation between the different methods and the other prognostic factors (IgVH mutational status, cytogenetics) that will be assessed in all patients enrolled by the study project. *Supported by Regione Veneto "Ricerca Sanitaria Finalizzata" and "Fondazione G. Berlucchi per la Ricerca sul Cancro".*

Table 1.

Anti-ZAP-70 mAbs	*Positive Control (cut-off 20%)*	*Isotype Control (cut-off 20%)*	*MFI Ratio (T/B-CLL) (pos < 4)*
1E7.2	74%	39%	100%
2F3.2	26%	30%	52%
SBZAP	13%	56%	35%

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HAIRY CELL LEUKEMIAS WITH UNMUTATED IGHV GENES DEFINE THE MINOR AGGRESSIVE SUBSET UNRESPONSIVE TO SINGLE AGENT 2CDA

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Hairy cell leukemia (HCL) is generally responsive to single-agent 2-Chloro- desoxiadenosine (2CdA) and only a minority of patients are refractory and with poor prognosis. HCL generally express mutated (M-HCL) and in a minority of patients unmutated immunoglobulin heavy chain variable region (IGHV) genes (UM-HCL). In a multicenter clinical trial in newly diagnosed HCL receiving subcutaneous 2CdA, we prospectively investigated the correlation of response to treatment with clinical parameters at presentation (anemia, leucocytosis, thrombocytopenia, hairy cell index, splenomegaly) and tumor IGHV status. Fourty of 47 patients responded to subcutaneous 2CdA (33 CR, 7 PR), while 7/47 patients demonstrated no (NR 6/7) or minor (mR 1/7) response. Six of 7 NR+mR patients rapidly progressed after treatment (median time to progression 8 months) and required new treatments. Responses and toxicity appeared independent of treatment schedule. Analysis of response prognosticators revealed unmutated IGHV gene status (6/7 NR+mR 1/40 CR+PR, $p=1.1\times10^{-8}$), large splenomegaly (6/7 NR+mR 4/40 CR+PR, $p=6.3\times10^{-6}$) and leukocytosis (5/7 NR+mR 3/40 CR+PR, $p=3.2\times10^{-5}$) as strong predictors of response. IGHV mutational status correlated significantly with leukocytosis (4/7 UM-HCL 4/40 M-HCL, p=0.002) and splenomegaly (5/7 UM-HCL 5/40 M-HCL p=0.0004), to suggest a more aggressive disease and a higher tumor burden at presentation in the UM-HCL. Also, IGHV ($p=2.5\times10^{-7}$), leukocytosis ($p=2.6\times10^{-7}$) and splenomegaly ($p=1.4\times10^{-5}$) predicted shorter progression free survival after 2CdA (median 9, 9 and 20 months, respectively). However, only 1/7 M-HCL with leukocytosis and/or splenomegaly scored resistant and progressed after treatment, while only 1/7 UM-HCL responded to treatment and did not progress, to suggest that unmutated IGHV status was the major determinant of resistance to treatment and of bad behavior in HCL. Specific analysis of the refractory patients by genome-wide DNA profile (250 K Affimetrix SNP array) and p53 mutational status revealed that only the one refractory M-HCL had 17p(p53) loss and that only 1/6 NR+mR UM-HCL had p53 mutation, to suggest mechanisms of response independent of specific genomic aberrations or p53 mutational status in UM-HCL. Our data suggest a strong correlation of IGHV-mutational status with treatment-response and clinical behavior and provides a new major element of concern for treatment choice in HCL.

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CD49D PROTEIN EXPRESSION REFINES THE PROGNOSTIC IMPACT OF THE TUMOR LOAD MARKERS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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CD49d/alpha4 integrin is a molecule functionally mediating cell-cell interaction through the binding with fibronectin or vascular cell adhesion molecule-1 (VCAM-1). Recent studies by our group (Zucchetto, 2006; Gattei, 2008) suggested that high CD49d expression was an independent prognosticator both for overall survival (OS) and time to treatment in B-cell chronic lymphocytic leukemia (B-CLL). Furthermore, it has been demonstrated that intermediate/high (int/high) modified Rai stages (mod-Rai), higher serum soluble CD23 (sCD23) and beta2-microglobulin (B2M) levels indicate great disease burden and poor prognosis. The aims of our study were: 1) to correlate CD49d with other biologic prognostic factors, 2) to determine progression-free survival (PFS) upon CD49d, 3) whether CD49d could predict varied outcome within a tumor burden subgroup defined by mod-Rai or sCD23 or B2M; and finally, 4) to test the independent prognostic value of CD49d. Therefore, we investigated 204 pts, median age 65 years (range 37-87), 105 males and 99 females. With regard to mod-Rai, 64 pts had a low stage and 140 an int/high stage. CD49d was performed by flow cytometry fixing a cut-off value of 30%. CD49d$^+$ pts were 94/204 (46%). CD49d >30% was associated with an int/high mod-Rai stage (p=0.0005), with marked lymphadenopathy and/or splenomegaly (p=0.00002) and higher sCD23 (p=0.0003). Noteworthy, a close association was found between higher CD49d and higher CD38 (38/47; p<0.0001). A significant shorter PFS was observed in CD49d+ pts (22% *vs.* 50% at 12 years; p=0.00001) as well as in int/high mod-Rai pts (23% *vs.* 82% at 12 years, p<0.00001), in higher B2M pts (19% *vs.* 52% at 12 years; p<0.00001) and in higher sCD23 pts (7% *vs.* 59% at 12 years, p<0.00001). In order to refine the prognosis for PFS, we investigated CD49d within int/high mod-Rai (140 pts), lower sCD23 (126 pts) and lower B2M (117 pts) subsets. As a matter of fact, CD49d$^+$ pts showed a shorter PFS both within the int/high mod-Rai (15% *vs.* 35% at 12 years, p=0.0001; Figure 1), within lower sCD23 (40% *vs.* 70% at 12 years; p=0.004) and within lower B2M subsets (38% *vs.* 65% at 12 years, p=0.01). In multivariate analysis of PFS, ZAP-70 (p=0.0001), B2M (p=0.0003) and CD49d (p=0.01) resulted to be independent prognostic factors. Therefore, CD49d, determined by flow cytometry, is a promising prognostic parameter in B-CLL and refines well the prognosis for PFS in combination with the tumor load markers such as mod-Rai, B2M and sCD23.

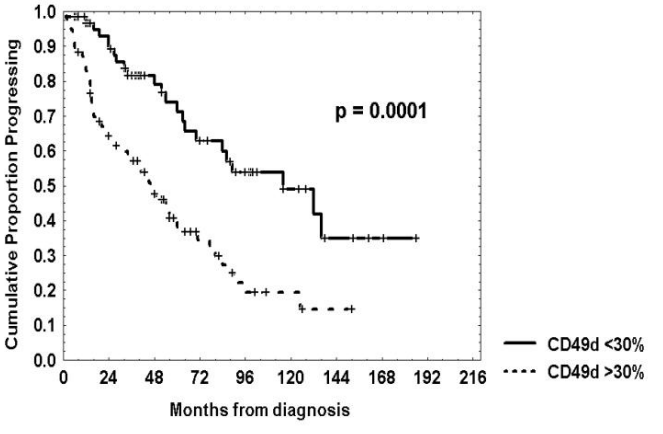

Figure 1. Progression-free survival within int/high mod-Rai stage.

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THE PROLIFERATIVE RESPONSE TO CPG-ODN STIMULATION PREDICTS DISEASE PROGRESSION IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Chronic lymphocytic leukemia (CLL) B-cells in some cases exhibit marked proliferation following stimulation with unmethylated CpG-oligodeoxynucleotides (CpG-ODN), that occurs primarily in leukemic B-cells from patients with progressive disease and unmutated IgVH genes. We correlated this proliferative response, IgVH, CD38, ZAP-70 and cytogenetic abnormalities to progression-free survival (PFS) to detect the prognostic value of a new biological marker. The proliferative response to CpG-ODN stimulation was investigated by analysis of [3H] thymidine incorporation in 65 CLL patients (39 male, 26 female, 65 yy median age, 49 Binet stage A, 12 B and 4 C). We evaluated the response based on the stimulation index (SI) (cpmCpG/cpmMedium). SI > 3 was indicative of proliferative response. Thirty-one cases were proliferating cases and 34 non-proliferating. About IgVH, 33 cases were mutated and 29 unmutated. Among 63 tested cases, 14 were CD38 positive and 26 ZAP-70 positive. FISH analysis was performed in 59 patients, 49 patients were low-risk and 10 high risk (Table 1). Proliferating subset had a significantly shorter median PFS (44 months *vs.* 87 months, p = 0.002). By univariate analysis, mutated IgVH genes (p=0.001), ZAP-70 expression (p=0.009) and proliferative response to CpG ODN stimulation (p=0.002) were significantly associated with longer PFS. By multivariate analysis the only independent variable associated to longer PFS was mutated Ig VH genes status (HR=3.553; 95% CI: 1.628-7.751). Kappa test showed the strongest grade of agreement between proliferative response to CpG ODN stimulation and IgVH genes mutational status (k=0.47, p<0.0001). We evaluated PFS according to proliferative response and VH gene mutation status. The combination of non-proliferating/mutated cases (38.7%) had a significantly longer median PFS (not reached at 199 months); non-proliferating/unmutated cases (11.4%) (60 months); proliferating/mutated cases (14.5%) (60 months); proliferating/unmutated cases (35.4%) (30 months), (Logrank=15.249; p=0.002; Breslow=14.978; p=0.002). These data confirm that the proliferative capacity of leukemic cells is an important determinant of the clinical course in CLL, strongly correlated with adverse biological prognostic features, in particular with unmutated IgVH genes. A new biological information emerged by combination of IgVH and CpG. Patients with CpG non proliferating and IgVH mutated genes, about 35%, can be considered as super stable patients.

Table 1.

Biological parameters (cut-off)		*CpG-ODN proliferating*	*CpG-ODN non proliferating*
IgVH (98%)	Unmutated	22 (71%)	7 (22%)
	Mutated	9 (29%)	24 (78%)
CD38 (30%)	Positive	11 (35%)	3 (9%)
	Negative	20 (65%)	21 (91%)
Zap70 (20%)	Positive	17 (55%)	9 (29%)
	Negative	14 (45%)	23 (71%)
FISH (HR: 11q-;17p-) (LR: +12; normal; 13q)	High risk	8 (29%)	2 (7%)
	Low risk	20 (71%)	29 (93%)

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CXCL8 AND INTERLEUKIN 6 IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA ARE UP-REGULATED THROUGH THE ACTIVITY OF CXCL12

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Interactions between stromal and tumor cells in the microenvironment play a key role in B-cell chronic lymphocytic leukemia (B-CLL) onset and progression. This complex cross-talk is based on cytokine release and intercellular contacts that contribute to regulate the mechanisms of cell survival and apoptosis. Among the cytokines involved in this network, CXCL8/IL-8 and IL-6 are particularly noteworthy as potential pro-survival molecule able to decrease apoptosis in B-CLL cells. In this study, we explored whether the CXCL12 chemokine of stromal origin can regulate the expression of CXCL8 and IL-6 in B-CLL cells. We analyzed primary peripheral blood B-cells from 19 B-CLL patients. Engagement of CXCR4 expressed by B-CLL with its own ligand CXCL12 induced a significant increased production of CXCL8 (17/19 cases) and IL-6 (15/16 cases) but was ineffective on IL-1, TNF, or IL-10 production, as simultaneously measured by cytometric bead array kit and flow cytometry analysis. The average fold-induction±SD was 3.2±1.4 (range 1.5-6.2) for CXCL8 and 3.9±2.6 (range 1.4-11) for IL-6. Real time PCR analysis confirmed the CXCL8 and IL-6 increased expression also at the level of mRNA. We then investigated the role of the mitogen activated protein kinases (MAPK) p38 and JNK as well as NF-kappaB pathways that can contribute to regulate CXCL8 and IL-6 production in some cells. To this aim, we used SB203580, a compound that specifically blocks p38 enzymatic activity, SP600125, a potent and selective inhibitor of JNK, and MG132, which selectively blocks proteasome and inhibits NF-kappaB activation. Neutralization of p38 activity, but not JNK, suppressed both constitutive and inducible production of CXCL8 and IL-6, thus suggesting that the p38 pathway is required for the expression of both cytokines in B-CLL. In contrast, the blockage of NF-kappa B activation inhibits the production of IL-6 but not CXCL8. Our data show that CXCL8 and IL-6 are physiologically regulated by the CXCL12/CXCR4 axis and suggest that regulation of both cytokines requires the p38 MAPK activity whereas NF-kappa B is necessary only for the expression of IL-6. Since CXCL8 as well as IL-6 have been implicated in progression of B-CLL disease, our findings suggest that, by up-regulating these cytokines, the CXCL12/CXCR4 axis may contribute to the pathogenesis of B-CLL. *Supported by Regione Veneto Ricerca Sanitaria Finalizzata and Fondazione G. Berlucchi per la Ricerca sul Cancro.*

P148

REDUCED INTENSITY/NON-MYELOABLATIVE ALLOGRAFTING FOR HIGH RISK CHRONIC LYMPHOCYTIC LEUKEMIA

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Refractory and high risk chronic lymphocytic leukemia (CLL) represents a clinical challenge despite new treatment developments. Until recently, allogeneic stem cell transplantation (alloSCT) has been infrequently used due to its toxicity. However, it has been associated with a potent graft-vs.-leukemia (GVL) effect with prolonged disease-free survival in surviving patients. The demonstration of the role of the GVL effect has led to the exploration of new conditioning regimens characterised by reduced toxicity and preserved GVL. We report our experience on 12 CLL patients (median age 50, r. 44-57) who underwent related (no.10) or unrelated alloSCT (no.2), all refractory to at least 2 lines of previous therapies, with chromosome 17 deletion (no.8), or $CD38^+$, $ZAP70^+$ or unmutated variable regions of the immunoglobulin heavy chains (no.2). Nine/12 were on therapy within 3 months before alloSCT. At the time of alloSCT, 5 patients were in partial remission (PR) and 7 had stable disease. Conditioning regimens were fludarabine (90 mg/m^2) with 2 Gy total body irradiation (no.7), or total lymphoid irradiation and thymoglobulin (TLI-ATG) (no.1) or thiothepa, cytoxan, melphalan (no.4). Only four patients developed acute graft-versus-host disease (GVHD), and 5/12 experienced chronic extensive GVHD. After a median follow-up of 19 months (range 2-38), median overall survival has not been reached and event-free-survival was 11 months (r. 2-34). Two patients died: 1 from fungal infection and 1 from neurotoxicity. Disease response in 10 evaluable patients was as follows: 3/10 complete remission (CR), 5 PR, 2 showed stable disease. At follow up, 3 patients progressed (2 progressions were detectable by flow-cytometry only). Two/12 patients received a second reduced-intensity alloSCT from the same donor because of disease progression (in continuous PR 29 months after the second alloSCT) and graft rejection (full donor engraftment 3 months after the second alloSCT). Despite the small number, we observed that high risk CLL is susceptible to GVL effects with acceptable toxicity. Thus, given the lack of valid therapeutic options in this patient population and the development of new promising non-myeloablative regimens (i.e. TLI-ATG) that reduce the incidence of GVHD, prospective studies to investigate the role of alloSCT in CLL are imperative.

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CALCIUM SIGNALLING EVALUATION IN RESIDUAL T LIMPHOCYTES FROM B-CLL PATIENTS

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A functional T defectiveness, including a reduced response to proliferative stimuli, has been reported over the paste two decades. One of the early biochemical event that follows T-cell recptor (TCR) engagement is the activation of the enzyme phospholiphase Cgamma1 (PLCgamma1) that stimulates the production of inositol-1-4-5-triphosphate (IP3) and this molecule, in turn, induces a sustained intracellular calcium elevation that is critical for the activation of IL-2 promoter by the calcineurin pathway, and for cell proliferation. Since this biochemical event is crucial for productive cell activation, in the present study we analyse the calcium release in residual T lymphocytes of B-CLL patients to investigate a possible defect in this pathway that may contribute to explain the unresponsiveness condition that characterize T cells in this disorder. The study was performed on peripheral blood mononuclear cells from 17 B-LLC patients and 10 healthy volunteers by flow cytometry (FACSCalibur, Becton Dickinson) using the fluorescent probe FLUO3-AM. FLUO3-AM signal was analysed in T cell population identified by FSC and SSC and $CD5^+/CD20^-$combination, and the results were expressed as a ratio between median FLUO3-AM intensity value (MFI) following TCR stimulation and ionomicin stimulation (maximal calcium raise). Our data show a reduction of intracellular calcium increment in T lymphocytes from all B-CLL cases, in fact the median value of MFI recorded in B-CLL and in normal control was 48 (range 35-79) and 87 (60-148) respectively. Ionomycin calcium release was similar in T cells from patients and donors (median MFI values were 198 and 158 respectively) demonstrating that the differences observed in patients and donors following TCR stimulation were TCR specific. T cells from 13/17 B-CLL patients showed also a reduced proliferative response, while 4/17 showed a response similar to healthy donors. In addition, the impairment of T cell calcium signalling was detected in patients at the diagnosis and with aggressive disease or at progression of the disease and do not seem to correlate with any of the biological parameter with prognostic relevance. In conclusion, these preliminary data suggest that the cytosolic calcium impairment may in part contribute to explain T cell proliferation deficit in B-CLL. Whether this alteration is due to an intrinsic T cell defect or to the interference of B neoplastic cells remains to clarified.

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VERBENA OFFICINALIS ESSENTIAL OIL AND CITRAL AS *IN VITRO* APOPTOTIC INDUCTORS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Preliminary evidence indicates that isoprenoids, a broad class of mevalonate-derived phytochemicals which are ubiquitous in the plant kingdom, may suppress, with great potency, the proliferation of tumor cells

as human breast adenocarcinoma (MCF7), human leukemia (HL-60) and human colon adenocarcinoma (CaCo2). Verbena officinalis L. (Verbenaceae), commonly known as vervain, is a medicinal plant which grows wild everywhere. Despite its pharmacological mechanisms of action is still unclear, V. officinalis essential oil has several traditional medicinal uses and is almost completely constituted of isoprenoid compounds. In this work we obtained the essential oil of V. officinalis from plants grown in the Garden of Medicinal Plants in the Campus of the State University of Salerno. The chemical composition of the volatile oil was achieved by GC and GC-MS methods. The essential oil and citral, its main constituent, were tested on 5 normal adult healthy subjects (1 F; 4 M; mean age 43 yrs; range 43-36 yrs) and 5 untreated patients suffering from chronic lymphocytic leukemia (CLL) (1 F; 4 M; median age 59 yrs; range 59-78 yrs). Mononuclear cells were isolated from peripheral blood samples by centrifugation on a Ficoll/Hypaque gradient and cultured at a cell concentration of 2,5×10^6/mL in RPMI 1640 culture medium in a humidified incubator 5% CO2 at 37°C. Cells were incubated for up to 24 hours with vervain essential oil or citral at different concentrations. The proapoptotic effect of the compounds was evaluated after three different times of incubation (4, 8 and 12 hours in dark conditions) by adding to cells annexin-V 5 microL and propidium iodide 5 microL (Annexin V-FITC Apoptosis Detection Kit 1, BD Pharmingen). FACSCanto flow cytometer (Becton Dickinson) was used to acquire and analyze samples. Both vervain essential oil and pure citral induced a significant apoptosis in CLL samples with respect to controls as shown in Figure 1. Necrosis was also found to increase with time, reaching the maximum after 24 hours. This data, despite preliminary and limited in number, clearly shown the proapoptotic properties of Verbena officinalis essential oil and citral in patients with CLL that was thought to be a disease that originating from antigen-stimulated B cells that escape normal cell death mechanisms. The apoptotic effect which is induced by this compund could be related to activation of the caspase 3.

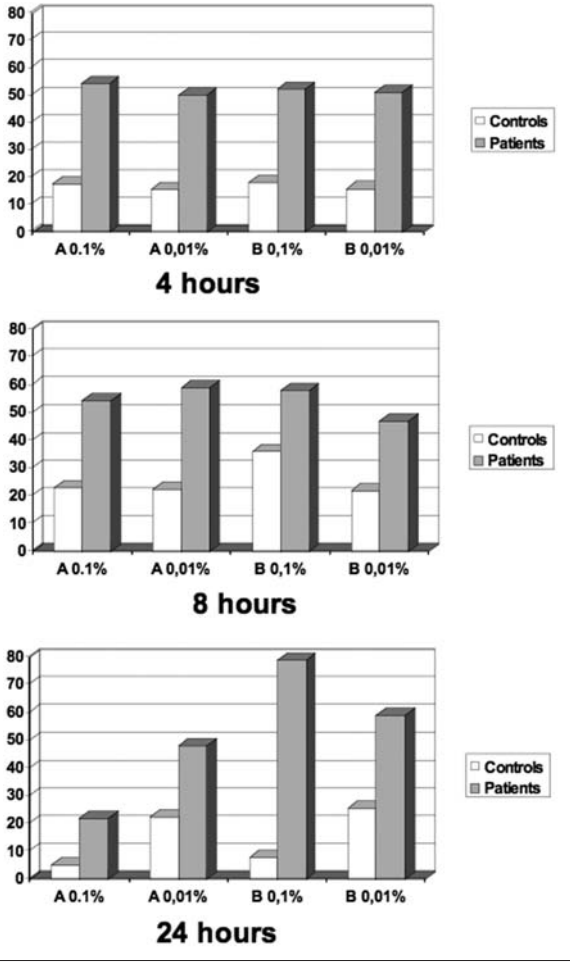

Figure 1. Officinalis essential oil (A) and Citral (B) induced-Apoptosis in CLL patients and controls at different times.

Lymphomas (I)

P151

ITF2357, A NOVEL HISTONE-DEACETYLASE INHIBITOR, IS EFFECTIVE AGAINST PERIPHERAL T-CELL LYMPHOMAS *IN VIVO*

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Background. Recently, gene expression profiling (GEP) indicated histone-deacetylases (HDAC) as potential therapeutic targets in peripheral T-cell lymphomas (PTCL) not otherwise specified (NOS), the commonest PTCL type. Consistently, phase II trials demonstrated the efficacy of some HDAC inhibitors (HDACi), including SAHA, which was approved for cutaneous T-cell lymphomas (CTCL) treatment. Aims and methods. We investigated the anti-tumour effects of ITF2357 (Italfarmaco, Italy), a novel hydroxamic acid HDACi, on PTCL primarily-cultured cells and cell lines (HH and FEDP), and in a xenografted mouse-model of CTCL. Cultured cells were incubated with different dosages of ITF2357 and SAHA (ranging from 0.5 to 2.5 μM). Cell viability, assessed by trypan-blue exclusion assay, cell-cycle progression, assessed by bromodeossiuridine assay, and apoptotic rate, determined by flow-cytometry analysis of annexin-V binding populations were determined at 48, 72 and 120 hours. Nude mice, injected with HH cells, received ITF2357 (10-20 mg/Kg, per os) for 14 days. Small animal PET was adopted for disease measurement and treatment response evaluation. Finally, GEP of cell lines exposed to ITF2357 and SAHA were generated to elucidate their mechanisms of action. Results. Cell viability of HH cells treated with ITF2357 ranged from 50% (0.5 μM, at 48 h), to <10% (0.5-2.5 μM, at 72-120 h), in comparison to untreated cells. Differently, cell viability of HH cells treated with SAHA ranged from 80% (0.5 microM, at 48-120 h) to 39% (2.5 μM at 48 h). Analogue effects were documented in FEDP and primarily-cultured PTCL cells. Conversely, viability of normal T-lymphocyte was not significantly affected. Interestingly, exposure to ITF2357 was associated to G0/G1 cell-cycle arrest and apoptosis induction. Finally, ITF2357 determined significant reduction of tumoral masses and survival benefit in a xenografted mice-model inoculated with HH cells. Conclusion. Taken together, these data demonstrate that ITF2357 is effective against PTCLs *ex vivo* and *in vivo*, by nominating it for clinical evaluation in this setting.

P152

18F-FDG SMALL ANIMAL PET EARLY DETECTS HUMAN HODGKIN LYMPHOMA XENOGRAFT IN NOD/SCID MICE

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Aim. To serially monitor over time the growth and variations of metabolism of human Hodgkin Lymphoma (HD) xenograft in NOD/SCID mice by 18F-FDG Small Animal PET. Material and *Methods.* Human cell line (DSMZ) L428 (HD) was subcutaneously injected (107cells/mouse in 100 uL PBS) in eight 6weeks-old NOD/SCID mice (Charles-Rivers) at right flank level. In all animals 18F-FDG Small Animal PET (GE eXplore Vista DR) was used to assess tumour growth at 1week (wk), 2wks, 3wks, 4wks, 5wks and 6wks after tumour cells injection. An early (4 days) PET scan was performed in a subgroup (4/8 animals). PET (18F-FDG i.v. injected dose: 20 MBq in <0,15 mL; uptake time=60 minutes static acquisition of 15 minutes, 1 bed position) was

performed under gas (Sevofluorane 5%, Oxygen 1L/min) anaesthesia (VetEquip Complete Anaethesia System, Pleasanton, CA). The residual dose in the syringe was measured to verify the effective dose injected. PET images were reconstructed iteratively (OSEM 2D) and read in three planes (axial, sagittal and coronal). PET was considered positive if any area of increased non-physiologic 18F-FDG uptake was observed. Semiquantitative analysis was performed using the target to background ratio: TBR= (Maximum counts in the most active tumour visible area)/(mean counts in the contra-lateral subcutaneous tissue). Pathology evaluation was performed in all cases. *Results.* The earliest tumour lesions at injection site could be identified by 18F-FDG Small Animal PET in 4/8 animals: at one week after cells injection in 3/8 L428-mice while one animal turned positive at 14 days. The remaining 4 mice were PET-negative at all times and did not develop any tumour. Mean TBR increased progressively up to 5 wks after injection while the last scan presented a slight decrease of the lesions metabolic activity (Figure 1). Pathology sections obtained at 6wks, confirmed PET findings in all cases: positive-PET mice presented a large tumour lesion with central necrosis, sparse Reed-Stemberg cells, and infiltrating adjacent muscles in two cases. Macroscopic and microscopic evaluation did not reveal any lesion in PET-negative mice. *Conclusions.* Small animal PET allowed the early selection of tumour bearing animals as early as 7 days after cells injection and was useful to monitor tumour metabolism over time.

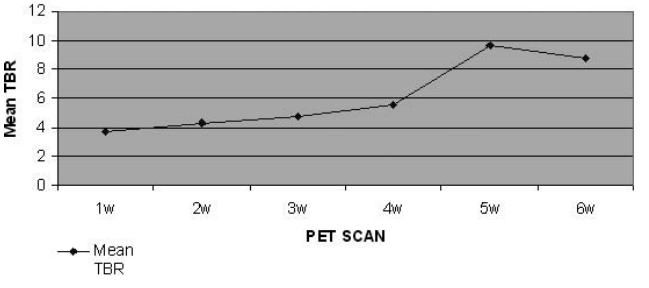

Figure 1. Mean TBR variations over time in NOD/SCID mice bearing Human Hodgkin Lymphoma.

P153

HIGH PLASMA DNA LEVELS CORRELATE WITH UNFAVORABLE CLINICAL CHARACTERISTICS AND PROGNOSIS IN LYMPHOMA PATIENTS

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Increased levels of plasma DNA have been observed in patients with a variety of cancers and correlated to clinical characteristics and prognosis. However, few studies have been focused on haematological malignancies. Our aim was to investigate the diagnostic and prognostic role of DNA plasma levels in patients with lymphoma. This study included 142 lymphoma patients at the diagnosis (45 pts with Hodgkin lymphoma (HL), 63 pts with diffuse large B cell NHL (DLBCL), 24 pts with follicular and 10 pts with mantle cell NHL) and 41 healthy controls. Plasma DNA was extracted using the QIAamp UltraSense Virus Kit (Qiagen, UK) for improving the isolation of fragmented DNA. A Q-PCR for the beta-globin gene was performed for each sample in triplicate using a calibration curve for the quantification of DNA levels. The coefficients of variability intra and inter-assay were both <0.01. Associations with patient characteristics and event-free survival (EFS) were analysed by standard statistics (STATA 10). In healthy controls plasma DNA ranged from 3 to 35 ng/mL, with a mean value of 13.9 ng/mL. Lymphoma patients had significantly higher DNA plasma levels, with mean values of 43.4 ng/mL in HL patients, 91.6 ng/mL in DLBCL, and 74.1 ng/mL in mantle cell NHL. DNA levels in patients with follicular NHL were not different from controls (22.9 ng/mL). Plasma DNA concentration correlated to several known adverse prognostic factors. Age >60 years, advanced stage of disease (III/IV) and LDH levels above normal range were associated with increased levels of plasma DNA, both analysing the total lymphoma group (p=0.018) and restricting the analysis to patients with either DLBCL (p=0.03) or HL (p=0.0001). In particular, in a multivariate analysis LDH levels were the strongest predictor for increased plasma DNA. The 2-years probability of failure-free survival was 56% (95%CI, 36-72%) in patients with elevated plasma DNA, while in patients with normal DNA levels was 90% (95% C.I., 79-95%) (p=0.001). Including age, LDH levels and stage into a multivariate analysis and stratifying for histotype and treatment, high plasma DNA levels were an independent risk factor for inferior EFS (HR: 3.3, 95%CI: 1.2-9.4). Moreover, in HL patients, high plasma DNA levels correlated with presence of necrosis and with NS2 histotype (p=0.007 and p=0.03, respectively). Our results suggest that plasma cell-free DNA may become a new promising biomarker with prognostic impact in patients with lymphoma.

P154

SAFETY AND EFFICACY OF BENDAMUSTINE WITH OR WITHOUT RITUXIMAB IN THE TREATMENT OF HEAVILY PRETREATED PATIENTS. A MULTICENTER RETROSPECTIVE STUDY

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Bendamustine is an alkylating agent with a nitrogen mustard group and a purine like benzimidazol group. Recently this drug was introduced in Italy and it was used in patients (pts) pretreated to test its efficacy and safety. We analized all pts treated in six haematological Italian centers with Bendamustine alone or in combination with Rituximab. Pts who have received at least one complete cycle were evaluated for response and toxicity, pts ongoing were not evaluated for response but for toxicity. The treatment consisted of: Bendamustine 60-90 mg/m^2 days 2,3 alone or in combination with Rituximab 375 mg/m^2 day 1, every 21 or 28 days. 38 pts were analized, the diagnosis were: 2 myeloma, 16 CLL/lymphocytic lymphoma, 6 diffuse large B cell lymphoma, 6 follicular lymphoma, 4 mantle cell lymphoma, 2 marginal lymphoma, 1 Hodgkin's disease and 1 Peripheral T cell lymphoma. Pts were heavily pretreated, the median number of previous treatments was 3 (range 1-8). Thirty-one pts were previously treated with Rituximab and 8 performed an autologous transplantation. The pre-treatment condition was: 12 relapsed pts, 6 with refractory disease and 20 with a progressive disease. The median number of Bendamustine cycles was 3 (range 1-8), 6 pts were on treatment. Thirty-two patients were evaluable for response: 7 complete remission, 14 partial response with an overall response rate of 66% and 11 non responders. No differences were observed according to Bendamustine dosage or scheduling. Only one patient with DLBCL obtained a CR and the other five were non responders, no pts with myeloma, Hodgkin and T cell lymphoma showed a response. The best results were obtained in 10 evaluable pts with indolent lymphoma (4 CR and 6 PR) and in 9 evaluable CLL (1 CR and 6 PR). Two evaluable pts with mantle cell lymphoma obtained a response (1 CR and 1 PR). With a median period of observation of 4 months 71% of pts are alive. After 130 cycles the extrahematological toxicity was mild and the hematological toxicity was trombocytopenia grade 4 in 2 pts and neutropenia grade 4 in one patient. In conclusion this retrospective study shows that treatment with Bendamustine alone or in combination with Rituximab is a safe and efficacy regimen in a subset of pluriresistent pts. This data shows also that the best results could be obtained in indolent lymphoma and incouraging data in mantle cell lymphoma. Pts with DLBCL were refractory and too elderly to extrapolate a lack of efficacy in this subset.

P155

HBV AND NON HODGKIN'S LYMPHOMA

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Background. In the general Italian adult population HBsAg positivity is <2%. Recently, a high prevalence of serologic markers of HBV has been described in NHL patients. During chemotherapy for NHL, HBV infection can reactivate in both HBsAg-positive patients and HBcAb-positive, anti-HBs-positive/HBsAg-negative individuals, the latter group to be considered as potential carriers of occult HBV infection. HBV reactivation may cause hepatitis, hepatic failure and even death. Pre-emptive treatment with Lamivudine (LAM), is now strongly recommended during the course of chemotherapy for hematological malignancies in HBsAg-positive patients. However, the management of HBcAb-positive, anti-HBs-positive/HBsAg-negative patients has not been clearly defined. Also the duration of LAM prophylaxis and the management of HBV reactivation under LAM treatment are still open questions. We aimed our study to investigate these points in a consecutive series of Italian patients affected by NHL. *Materials and Methods.* All patients diagnosed with NHL at our Institution from January 2005 to January 2008 were screened for HBV serology: HBsAg, anti-HBs, anti-HBc, HBeAg, anti-HBe. Patients were also tested for HCV antibodies. Pre-emptive treatment with LAM 100 mg/day was started 3-4 weeks before the starting of chemotherapy in all HBsAg-positive and in all HBcAb-positive, anti-HBs-positive/HBsAg-negative. Quantitative HBV-DNA analysis was also carried out, in HBV-carriers, every two months after the start of treatment. *Results.* Fourteen out of 133 patients (10.5%) had serological signs of HBV exposure. Four out of 14 were also co-infected by HCV (28%), while HCV infection rate was 4.5% in the all sample. In six out of 14 patients (42%) HBS-Ag was negative, being anti-HBC (5 patients) and anti_HBs (1 patient, no history of vaccination), the only positive marker of cryptic infection. Follow-up from the starting of LAM ranged from 2 up to 27 months (median 12). Five of the 8 HBsAg positive patients (62%) became HBV-DNA positive during follow-up at 26, 17, 14, 8 and 7 months after the starting of chemotherapy despite LAM prophylaxis. Two patients also showed an increase of serum transaminases (up to 5X normal). In these 5 patients Adefovir 10 mg/day was added on to LAM, with disappearance of HBV-DNA and regression of the biochemical signs of hepatitis. None of these patients had to discontinue chemotherapeutic protocol because of HBV reactivation. No liver-related death occurred. None of the HBcAb-positive, anti-HBs-positive/HBsAg-negative patients developed HBV reactivation during the period of observation. No side effects related either to LAM or ADV were registered. *Conclusion.* Prevalence of HBV infection is high among patients diagnosed with NHL as compared to the prevalence in the Italian adult population. Reactivation of HBV infection develops, despite LAM prophilaxys, in a substantial proportion of HBsAg-positive patients. It can represent a serious threat to the patient and to the completion of the chemotherapeutic regimen. Since HBV reactivation might develop also several months after the stopping of chemotherapy, long term LAM treatment and serologic follow-up for HBV reactivation is recommended. In case of break-trough HBV reactivation under LAM treatment, ADV add on seems to be an effective policy. In our series none of the HBsAg-neg/anti-HBc-pos patients developed HBV reactivation under LAM prophilaxys. More studies are necessary to define 1) the correct management of HBcAb-positive, anti-HBs-positive/HBsAg-negative patients, 2) the duration of LAM treatment and of HBV surveillance after LAM treatment discontinuation, and 3) the management of antiviral drug resistance development in this setting.

P156

NON MYELOABLATIVE ALLOGENEIC STEM CELL TRANSPLANTATION IN PATIENTS WITH HIGH RISK LYMPHOMA: A SINGLE CENTRE EXPERIENCE

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From December 2000 to March 2008, 40 patients (22 females and 18 males) median age 44,5 years (range 18-66) underwent NST because of high risk Hodgkin Disease 10 cases , non Hodgkin Lymphoma 22 cases and Chronic Lymphocytic Leukaemia 8 cases. Disease status at transplant was as follow: 10 in complete remission 19 in partial remission and 11 in progression. In 35 cases, grafts were mobilized from HLA identical sibling donors, in 5 case by a matched unrelated donor (MUD) bone marrow. Conditioning regimens consisted of Fludarabine, Thiotepa and Cyclophosfamide in 22 cases, Total Limphonodal Irradiation and ATG in 7 cases, Fludarabine and Cyclophosfamide in 5 cases, Fludarabine and Thiotepa, Fludarabine and Melphalan, Thiotepa and ATG, TBI and Fludarabine ,in 1 case, Campath-1, Fludarabine, Melphalan and TBI in 3 cases., Cyclosporine–A and Methotrexate were used as GvHD prophilaxys in 31 cases, in three Campath-1 and Moftil micofenolate and in 7 cases Cya and Moftil micofenolate were used. A mean number of 5.48×10^6/Kg $CD34^+$ cells (range 3,4-7.7) were infused. The median number of red blood and platelet transfusion were 5,5 (range, 0-22) and 4 (range, 0-32, respectively. Only 12 patients experienced mucositis. 15 patients had fever . The median time to platelet and neutrophil counts higher than 20×10^9/L and 0.5×10^9/L were 11 (range, 6-19) and 21 days (range, 12-39), respectively. 4 patients developed a WHO grade 1 cutaneous acute GvHD, , 2 patient W.H.O. grade 4 liver acute GVHD, After a median follow up of 38,4 months, (range 8-91), 28 patients are Alive.Four patients experienced cronic GVHD Eigth transplant related deaths were documented. Three patients died for disease recurrence at 10 , 17 and 36 months post-transplant respectively, One patient died for Lymphoma non EBV related at 11 months from transplant. Chimerism study showed a full donor situation in 19 cases. In conclusion, NST is feasable and should be considered for high risk lymphoma patients.

P157

THE RELATIONSHIP BETWEEN ABSOLUTE LYMPHOCYTE COUNT WITH OS AND PFS IN PATIENTS WITH NON HODGKIN LYMPHOMA UNDERGOING AUTOLOGOUS STEM CELL TRANSPLANTATION

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Introduction. Autologous stem cell transplantation (ASCT) may improves survival of non Hodgkin Lymphoma (NHL) patients (pts). Several studies have reported clinical factors that are predictive of poor outcome. In this study, we have conducted retrospective analysis of factors that could predict OS and PFS in a setting of pts with NHL. Patients and Methods. We assessed 36 pts (19 males and 17 females) with median age of 51.8 years (r.22.5-69.3) and several histological types of NHL. The median number of cycles of chemotherapy was 3 (r.1-6). Stem cell mobilization consisted of various types of chemotherapy associated with G-CSF 5 microg/kg/day started from +3. Thirteen out 36 pts were in complete remission (CR) at the transplant time; the others showed an advanced status of disease. The main conditioning regimens were BEAM (24 cases) and Melphalan plus Mitoxantrone (7 cases). Variables analyzed with regard to PFS and OS were age, sex, status at transplant, $CD34^+$ cell infused, engraftment times, infections treated, ALC pre apheresis, at days +15 and +90 post-ASCT. Results. Median number of $CD34^+$cells infused was 4.4×10^6/kg (r.2.6-7.1). The median time to reach PMN >500/mL and PLT >30.000 was 11 days (r.9-14) and 13 days (r.9-

22), respectively. Twenty-five pts had an infection disease treated with empirical antibiotic therapy. After a median follow-up of 19.5 mo. (r.4-113), 26 (72.2%) and 22 pts (61.1%) were alive and in CR, respectively. The multivariate analysis showed that the number of salvage chemotherapy (p=0.028) and ALC at the time of apheresis (p=0.05) were the predictive factors influenced OS. ALC at +15 (p=0.02) is the only variable positive associated to PFS. We found that the value of ALC above 4×10^3/microl at apheresis and $0{,}95\times10^3$/microl at +15 days was the cut-off of significance in term of positive impact on OS and PFS, respectively. Conclusion. Our study demonstrates that the number of ALC on day 15 in NHL patients was better related with PFS while probably the number of ALC at the apheresis was correlated with OS. A threshold number of $CD34^+$ cells should not be the only parameter considered for an adequate PBSC collection, probably the number of lymphocytes should be aimed for as well. Finally, this study may contribute to support the hypothesis that the immune system may be important in the disease control, but to better understand these results, an immunological study is needed to evaluate the various subset of lymphocytes.

P158

POSSIBLE EFFICACY OF LAMIVUDINE PROPHYLAXIS TO PREVENT HEPATITIS B VIRUS REACTIVATION DUE TO RITUXIMAB REGIMENS IN HEPATITIS B VIRUS CARRIERS WITH B CELL LYMPHOPROLIPHERATIVE MALIGNANCIES

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Reactivation of Hepatitis B virus (HBV) is a recognized potentially fatal complication in patients undergoing chemotherapy for hematologic malignancies. Rituximab – based regimens induce long lasting effects on B lymphocytes increasing the probability of HBV reactivation. Lamivudine prophylaxis reduces the risk and the severity of HBV reactivation in patients with B lymphoprolipherative diseases. We report preliminary data about of a prospective study aimed to assess pre-emptive lamivudine in preventing HBV reactivation due to rituximab therapy. Four patients HBV positive at the diagnosis - three with diffuse large B cell lymphoma (DLBCL) and one with B-Cell chronic lymphatic Leukemia (B-CLL)-consecutively admitted to our Institution, received prophilaxis with lamivudine 100 mg once daily from at least seven days before starting chemotherapy to six monthes after chemotherapy completion. Three patients received R-CHOP and one R-Flu-Cy chemotherapy regimens. Patients HBV carriers, identified at the diagnosis by HBs antigen and/or HBc antibody positive screening were controlled for HBV DNA copies levels and liver function before every rituximab-chemotherapy cycle administration, and monthly after rituximab-chemotherapy completion. No hepatitis B virus reactivation has been observed, lamivudine was well tolerated and chemotherapy was administered as planned. *Conclusions.* based on our limited preliminary experience, lamivudine prophylaxis may be efficacious to prevent HBV reactivation in HBV carriers with B cell lymphoprolipherative malignancies receiving rituximab containing chemotherapy regimens; a close monitoring need to be performed during and after treatment.

P159

A CASE OF COMPOSITE LYMPHOMA CHARACTERIZED BY MULTIPLE RELAPSE AND OSTEONECROSIS OF LEFT FEMORAL HEAD DUE TO STEROID TREATMENT

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A 44 years-old man was admitted to our day-hospital with a 3-month history of left cervical lymphoadenomegaly. The histological examination of nodal biopsy diagnosed an Anaplastic Diffuse Large B Cell Lymphoma (DLBCL), $CD20^+$ and $CD30^+$. Clinical stage (CS) was IIIA aa-IPI 1. The patient achieved a PET/CT-documented complete remission (CR) following 6 courses of Promece-Cytabom polychemotherapy. After a period of 4 months, a new left cervical lymphoadenomegaly appeared. A new nodal biopsy revealed a composite lymphoma (CL). Morphological findings showed the co-existence of anaplastic DLBCL ($CD30^+$, $CD20^+$, $CD15^+$, $CD79A^+$) and Hodgkin's lymphoma (HL) ($CD45^+$, $CD15^+$). The CS at relapse was IIA, aaIPI 0 and IPS 1. Salvage treatment consisted of 6 courses of IGEV, with peripheral blood stem cells collection during the 3rd course. After the completion of the second-line treatment, the patient achieved a 2nd CR and refused autologous transplant. Unfortunately, three months later the patient newly developed an enlargement of left cervical nodes. Therefore, a new biopsy was performed and its histological examination showed a relapse of Anaplastic DLBCL without HL. After a subsequent treatment with involved field radiotherapy (30 Gy), the patient received a new PET/CT which showed a 3rd CR; in addition, an osteonecrosis of left femoral head was detected (confirmed by MNR). Actually, the patient is alive and in continuous CR after a follow-up of 21 months from the 2nd relapse. CL is a rare entity characterized by the presence of well demarcated Hodgkin's and non–Hodgkin's Lymphoma in the same organ or mass. The most frequent combination is HL and DLBCL. In particular, the lymphomas are defined synchronous if they are contemporaneously present and metachronous if the 2nd lymphoma follows the 1st in the time. The occurrence of CL furtherly supports the evidence of a common origin of Hodgkin's and non-Hodgkin's Lymphomas. CL has not a gold standard therapy today. We believe that these cases need the administration of a polychemotherapy that includes drugs effective both in Hodgkin's and non-Hodgkin-s lymphomas. With respect to osteonecrosis, we think that its onset in our patient is due to the cumulative dose of prednisone (5040 mg/m^2) included in the Promece-Cytabom regimen. Because this adverse effect may reduce the quality of life, the risk of its occurrence should be considered during the treatment planning of patients with lymphoma.

P160

AN UNUSUAL COMBINATION OF MULTIPLE MYELOMA AND FOLLICULAR LYMPHOMA

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In a 63-year old woman with a seric monoclonal component (MC) a smouldering IgG k Multiple Myeloma (MM) was diagnosed. Spine and pelvis MRI revealed a diffuse pattern of bone marrow (BM) involvement. Three years later the whole pattern was unchanged, with slight progression of spinal involvement, without osteolyses. The 18FDG PET-CT instead revealed hyperactivity in humeri, dorsal and lumbar vertebrae, ribs but, unexpectedly, even in the liver, spleen, and retroperitoneal and inguinal lymph nodes. Body RMI confirmed hepato-splenomegaly within nodular lesions and multiple abdominal lymphoadenopaties. At the BM trephine biopsy an interstizial follicular lymphoid infiltrate $CD79A^+$, $CD3^-$, $CD20^+$, $CD10^+$, $BCL6^+$, $BCL2^{+/-}$, high Ki67, coexisted with a monoclonal atypical plasmacellular population; the immunofluorescence showed positive staining with the same k antisera in the cytoplasm of plasma cells and in the centroblasts of the neoplastic follicules; bcl-2 rearrangement was detected: stage I MM coexisting with stage IV, grade III follicular NHL.

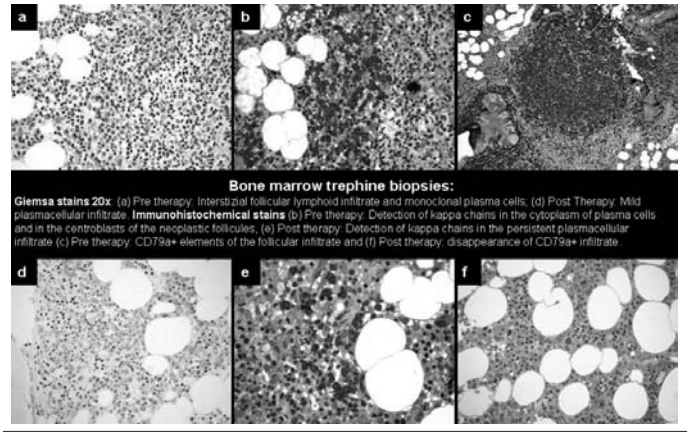

Figure 1.

After CHOP plus rituximab chemotherapy and radiotherapy towards lumbar spine (progressive disease in L3) the MC disappeared and a complete regression of the multiple lymphoadenopaties and the nodular lesions was obtained; the PET resulted negative; the BM showed a disappearance of the lymphomatoid infiltrate, but the persistence of a mild plasmacellular infiltrate. After one year the PET persisted negative, while a little seric MC reappeared. The patient is now being treated with quar-

terly rituximab and monthly zoledronic acid. The occurrence of two malignancies suggests a genetic predisposition, exposure to similar carcinogenic agents and a dysregulation of the immune system. A common stem cell, probably in the lymphoid germinal centre, may contain aberrations that induce the development of clonal diseases in a multistep process. Failure in the control of molecular processes may lead to B cell tumorigenesis for example through the generation of translocations into Ig gene. While it is well documented that immunotherapy with anti-CD20 is effective in B-cell NHL, its role in MM is undetermined and controversial. Recent studies suggest the existence of clonogenic CD20-positive precursor B cells in the disease even if the expression of CD20 by MM cells is heterogeneous (15% of patients). Some clinical trials show that rituximab elicits a partial response in 10% of $CD20^+$ MM patients and sometimes a disease stabilization.

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MUCOSA ASSOCIATED LYMPHOID TISSUE LYMPHOMA OF THE LUNG (BALTOMA): EXPERIENCE OF A SINGLE CENTRE

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Malt lymphoma of the of the lung (BALTOMA) is a very rare entity; in reality it represents only 3.6% of all extranodal lymphomas and 0.4% of all non-Hodgkin's lymphomas and occurs predominantly in men. The role of chronic infections in BALTOMA is still unknown. The 60-70% of patients with BALTOMA presents a localized disease (stage IE-IIE). Due to the low-grade nature of BALTOMA, patients are often asymptomatic. BALTOMA are frequently diagnosed incidentally by routine chest roentgenograms; the characteristic computed tomographic findings include airspace consolidation or nodules with air bronchograms. Abnormal karyotype with chromosome translocations is frequent in this lymphoma of lung. BALTOMA tends to remain localized for a long time and for this indolent evolution may be managed conservatively with limited resection, low toxicity chemotherapy, and immunotherapy with anti-CD20 or with low dose radiotherapy to obtain symptom control. A total number of three patients (2 female/1 male - median age of 66 years) were identified between January 1996 and December 2007 in our institution. At diagnosis the patients presented with unspecific symptoms which were further evaluated. The diagnosis was performed after chest X-ray and computed tomography scan. At presentation, two patients had stage IAE and one IIAE. In two cases was also performed a PET at presentation, which confirmed the exclusive location and during and after the treatment that showed the RC after the treatment. All patients have undergone lung biopsy for the histological diagnosis. One patient was treated with CHOP protocol and for progression with fludarabina, than with chlorambucile, rituximab and RT. One patient has achieved a marked regression of lung localization after treatment with rituximab as a single agent than a RP with chlorambucile. One patient obtained a RC with chlorambucile and rituximab. Two patients are living in RC and in good general condition. One patient has died by secondary gynaecological metastatic cancer but in RC from lymphoma. Due to indolent nature of BALT lymphomas, the prognosis is good, and the treatment option of choise is local resection for limited disease, followed by radiotherapy for advanced and disseminated disease, respectively. However optimal standard treatment for pulmonary lymphoma is not defined. The rituximab has an important role as single agent in these patients for low toxicity with significant activity on pulmonary lesion.

Lymphomas (II)

P162

ASSOCIATIONS BETWEEN PLASMA LEVELS AND PROMOTER GENOTYPES OF IL-10 AND IL-6 WITH PATIENT CHARACTERISTICS AND OUTCOME IN HODGKIN LYMPHOMA

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Cytokines produced by Reed-Sternberg cells and the surrounding tissue contribute to the biology of Hodgkin lymphoma (HL). Plasma levels of various cytokines, such as IL-6 and IL-10, have been associated to prognosis in HL. We have shown in a group of 184 HL patients that single nucleotide polymorphism (SNP) in the promoter region of cytokine genes can predict outcome in HL: homozygous carriers of the allele -592A in the IL-10 promoter and of the allele -174G in the IL-6 promoter had a significantly lower probability of failure-free survival (FFS) (Hohaus et al, Ann Oncol 2007). We now analyzed whether cytokine plasma levels are associated with promoter genotypes and other patients characteristics. IL-6 and IL-10 plasma levels were determined in 81 patients with HL and in 74 healthy controls, and associations to the polymorphic allele variants in the IL-10 gene (T-3575A, G-2849A, C-2763A, A-1082G and C-592A) and in the IL-6 gene (G-174C) were studied. IL-10 and IL-6 plasma levels were significantly higher in patients than in controls (IL-10, median 23.1 pg/mL in HL patients *vs.* <5 pg/mL in controls (p=0.01); IL-6 median 2.6 pg/mL in HL patients *vs.* <0.001 pg/ml in controls (p<0.001; Wilcoxon rank sum test). Cytokine levels were dichotomized by the receiver operating characteristics technique. IL-6 plasma levels higher than 9.5 pg/mL were related to inferior FFS and overall survival (OS) (p=0.002 and p=0.04), while IL-10 plasma levels higher than 65 pg/ml were associated with inferior OS (p=0.05). In the multivariate logistic regression analysis including patients and controls (n=154), the presence of HL (HR 2.7; 95% CI, 1.2-5.9), male gender (HR 3.3; 95% CI, 1.5-7.3), and the IL-10 -592 AA genotype (HR 3.1; 95% CI, 1.1-9.1) were associated with higher IL-10 plasma levels (>65 pg/mL). Analyzing only the patient group, stage IV disease (HR 7.3; 95% CI 1.8-29), male gender (HR, 3.7, 95% CI, 1.2-12) and the homozygous -592A allele in the IL-10 promoter (HR, 8.5; 95% CI, 1.6-46) resulted associated with elevated IL-10 plasma levels in the multivariable analysis. Advanced stage of disease was also related to elevated IL-6 plasma levels (HR, 4.0; 95% CI, 1.03-15), but no association was found between IL-6 plasma levels and the IL-6 -174 genotype. In conclusion, stage of disease reflecting tumor burden and cytokine promoter genotypes are independent factors which determine plasma levels of IL-10 in HL.

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RITUXIMAB AS TREATMENT FOR MINIMAL RESIDUAL DISEASE IN HAIRY CELL LEUKAEMIA: EXTENDED FOLLOW-UP

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Summary. Purine analogues have dramatically improved the outcome of patients affected by Hairy Cell Leukemia, although complete eradication of disease can be rarely achieved. In our Institution, between May 2002 and November 2006, twenty-seven HCL patients (25 male and 2 female; median age 60 years, range 39-72) were treated with anti-CD20 after a pre-treatment with Cladribrine. At the time of enrollment in the study, overall response after Cladribrine was 89% (complete remission 26%, partial remission 63%; three cases were unresponsive). The median time from the last 2-CdA infusion and the beginning of this protocol was 4.3 months (range 1,5-113 months). Minimal residual disease was evaluated by repeating PCR assays. All 27 cases were PCR-IgH positive after induction. Two months after the end of anti-CD20 therapy, negativity for IgH rearrangement was detected in 30% of cases. Moreover, Rituximab increased subsequently percentage of molecular remission up to 70% at the latest analysis (median follow-up 36 months, range 13-68). The complete molecular response appears to be persistent: indeed, a recent up-date showed that all 19 out of 27 patients retained the IgH-negativity with a median follow-up 36 months (range 13-68 months). These results not only confirm the therapeutic effect of Rituximab, but also its significant role in eradicating MRD in HCL.

P164

EXPRESSION OF CD200/OX2 IN HEMATOPOIETIC MALIGNANCIES: IMPLICATION FOR CLINICAL CELL ANALYSIS AND MONOCLONAL ANTIBODY IMMUNOTHERAPY

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CD200/OX-2 is a toleragenic protein expressed on normal blood cell subsets as B-cells and activated T-cells as well as on cancer stem cells from several solid neoplasms. CD200 ligand (CD200R) is localized on macrophages and dendritic cells and its activation cause a reduction in cytotoxic immune response. The availability of a monoclonal antibody (MAb) designed for patient therapy (Alexion, USA), renders this molecule particularly intriguing. We studied the expression of CD200 on neoplastic cells from various hematologic malignancies, id est acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), acute hybrid leukemia (AHL), lymphoproliferative disorders (LD) and multiple myeloma (MM), with the aim of exploring the role of anti-CD200 MAb as a diagnostic tool and as a new therapeutic option in hematologic oncology. We analyzed 127 samples (57 bone marrow aspirates, 57 peripheral blood and 13 FNAC) using six-color flow cytometry with PE-conjugated anti-CD200 and FACSCantoII flow cytometer (BD). Results regarding percent positive cases, percent positive cells (PPC), as well as mean fluorescence intensity (MFI) are reported in the Table 1. phoma (DLCL) and lymphoplasmocytic lymphoma (LPL).

Table 1.

Disease	*N*	*Positive cases N (%)*	*% pos cells (mean)*	*% pos cells (25^{th}-75^{th} percentile)*	*Mean fluorescence intensity median value)*	*Fluorescence intensity (25^{th}-75^{th} percentile)*
AML	28	19 (68)	30.5	5-48	293	134-532
APL	2	0 (0)	2.5	-	33	-
AHL	5	4 (80)	60.2	27-87	420	238-1194
ALL	6	5 (83)	58.2	43-83	819	273-1251
CLL/SLL	33	33 (100)	98.5	98-99	3463	2767-4680
LPL	4	4 (100)	58.7	48-66	536	351-782
MCL	11	8 (72)	43	3-71	665	20-835
FL	10	1 (10)	4.3	2-6	46	30-83
DLCL	7	2 (28)	34.4	4-86	60	10-880
MZL	7	4 (57)	44.7	4-85	617	44-958
HCL	4	4 (100)	84.5	68-99	1353	1150-5922
MM	9	8 (88)	48.9	13-78	3421	667-5716
T-NHL	1	0 (0)	1	-	18	-

CD200 positivity was assigned to cases with MFI higher than 256. Among acute leukemia samples, ALL and AHL displayed CD200 with higher PPC and MFI as compared to AML. Acute promyelocytic leukemia (APL) samples showed the complete absence of CD200. In AML we also studied the correlation of CD200 expression with that of a large number of antigens (N=40) on blastic population (Spearman's test). We found a significant (p<0.05) direct correlation of CD200 with CD7 (r=0.41), CD135 (r=0.44), CD44 (r=0.46), CD45RA (r=0.38) and an inverse correlation with CXCR-4 (r=-0.5), disclosing an association of CD200 expression with an *early blast cell* immunophenotype. As regards LD, differences in PPC and MFI between chronic lymphocytic leukemia (CLL/SLL) and mantle cell lymphoma (MCL) was found to be statistically significant (p<0.0001). In CLL, CD200 expression was constantly high, indicating a role of CD200 as disease hallmark. In conclusion anti-CD200 shows an emerging role as a diagnostic tool in clinical immunophenotyping and can be considered a therapeutic option especially in CLL/SLL, hairy cell leukemia (HCL), ALL and in selected cases of other hematologic neoplasms such as AML, MM, MCL, marginal zone lymphoma (MZL), large cell lym

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RITUXIMAB PLUS CHOP OVERCOMES BCL2 ASSOCIATED RESISTANCE TO CHEMOTHERAPY IN PATIENTS WITH DIFFUSE LARGE B-CELL NON HODGKIN'S LYMPHOMAS

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Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous entity and patients exhibit a wide range of outcomes. BCL2 protein expression has been associated with poor prognosis in patients with DLBCL The addition of rituximab to CHOP chemotherapy (R-CHOP) has led to a marked improvement in survival and has cast doubt on the significance of previously recognized prognostic markers. We performed a retrospective analysis of 111 patients with de novo DLBCL treated at our institute between 2000 and 2005, to assess the value of BCL2 expression in the era of immunochemotherapy. Histological diagnoses were established according to the REAL-WHO classification. HIV-associated lymphomas, transformed lymphomas, cases with central nervous system involvement, primary mediastinal and primary extranodal DLBCL were excluded. Tumors were considered positive when at least 50% of tumor cells expressed bcl-2 protein. All patients received CHOP every 3 weeks; 58 patients were treated with chemotherapy plus rituximab (R-CHOP) and 53 patients with chemotherapy alone (CHOP). There were 68 (61%) bcl-2^+ patients and 43 (39%) bcl-2- patients. The response rates for R-CHOP and CHOP were 71% and 59% (p<0.05) in bcl-2^+ patients and 74% and 72% (p=n.s.) in bcl-2- patients, respectively. At a median follow-up of 3 years, R-CHOP was significantly associated with a better overall survival than CHOP in bcl-2^+ patients (79% *vs.* 48%, p<0.05). In bcl-2^- patients there was no statistically significant difference in terms of overall survival (75% versus 68%, p=n.s.). In addition, R-CHOP was associated with significantly better progression-free survival rates than CHOP in bcl-2^+ patients (Figure 1 b:65% versus 38%, p<0.01) but not in bcl-2- patients (60% versus 40%, p=n.s.). Multivariate analysis confirmed the significant benefit on survival and progression-free survival of R-CHOP in bcl-2^+ patients.

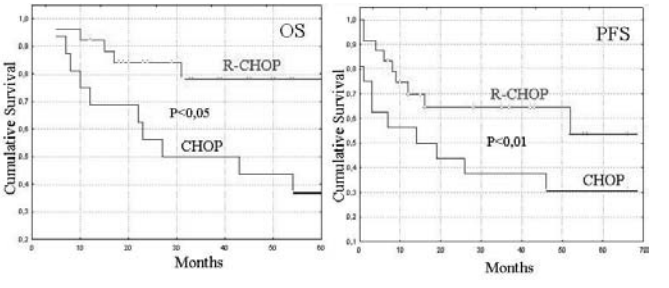

Figure 1. OS and PFS in bcl-2^+ patients with DLBCL.

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EVALUATION OF EARLY CARDIOTOXICITY USING BIOMARKERS IN LYMPHOMA PATIENTS TREATED WITH TWO DIFFERENT DOXORUBICIN CHEMOTHERAPIC REGIMENS

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Doxorubicin is extensively used in the therapy of lymphoid malignancies. However, its therapeutic value is limited by cumulative dose related cardiotoxicity. Recent data suggests that circulating biomarkers such as N-terminal pro-brain natriuretic peptide (NT-proBNP) and Troponin I (TROPI) are sensitive and specific predictors of cardiotoxicity. Elevated secretion of NT-proBNP has been associated with both left ventricular systolic dysfunction and diastolic fillings abnormalities in patients treated with anthracycline. The present study was conducted to compare the presence of early cardiotoxicity during the treatment in non Hodgkin's and Hodgkin's lymphoma patients treated respectively with the R-CHOP and ABVD protocol at the same cumulative doxorubicin dose of 300 mg/sqm. 33 non Hodgkin's lymphoma (NHL) patients, median age 58 (range;25-74), received 6 cycles of R-CHOP regimen and 32 Hodgkin's lymphoma (HL) patients, median age 29,5 (range;16-64), received 6 cycles of ABVD regimen. We prospectively evaluated serial TROPI and NT-proBNP measurements and echocardiographic parameters in the early detection of anthracycline induced cardiotoxicity. The value of plasma levels of NT-proBNP and TROP I were measured at baseline and before and after each cycle of therapy. Echocardiography was performed at baseline and after the fourth and the sixth cycle of chemotherapy. In all patients baseline echocardiographic parameters and biomarkers were normal. Both in NHL group and HL group, during the therapy, the TROPI value did not change significantly whereas an increase of NT-proBNP level was observed. In NHL group a transient increase of NT-proBNP level was observed in 6 patients (18%) after the first cycle of R-CHOP regimen at a cumulative doxorubicine dose of 50 mg/sqm; in HL group a transient increase of NT-proBNP was observed in 13 patients (26,5%), during the second-third cycle of ABVD regimen, after a cumulative doxorubicin dose of 100-150 mg/sqm and in 2 patients (4%) the values remained higher than baseline up to end of the chemotherapy. In HL group no significant impairment of left ventricular ejection fraction was observed whereas one patient from the NHL sub-group, with an increase of NT-proBNP value, showed a pathological drop of EF <50%. No cardiac event were documented in the two groups. Our data suggest that low doxorubicine doses of R-CHOP and ABVD regimen can cause a transit increase of NT-proBNP levels; nevertheless the peak of NT-proBNP compares earlier (50 mg/sqm) in CHOP group than in ABVD group (50-100 mg/sqm). Moreover in ABVD group no echocardiographic parameters impairment was observed during the therapy whereas in CHOP group one patient showed a pathological drop of EF <50% related to NT ProBNP increase. Further studies in larger populations with a longer follow-up are warranted to define the role of biochemical markers to reliably detect early myocardial injury during chemotherapy.

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SAFETY AND EFFICACY REDUCING GRANULOCYTE COLONY-STIMULATING FACTORS (G-CSF) VIALS IN R-CHOP14 SCHEMA

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Rituximab plus CHOP14 is increasingly used in the treatment of diffuse large B cell lymphoma (DLBCL). Some recent studies lead to the conclusions that intensified R-CHOP could be considered the standard of care for young patients with good prognosis DLBCL and the new standard for elderly patients with DLBCL (MInT trial and RICOVER 60 trial). Dose dense therapy is feasible with GCS-F support which is recommended for10 days. Starting from 2002 we have treated young patients with IPI:0-1 and elderly patients all affected by DLBCL. We prospectively decided to use 7 vials of G-CSF (from +5 to +11) in first ten patients and in absence of infections or delays the following patients were treated with 5 vials (from +7 to +11). Moreover if patients reached a number of leucocytes over 20.000/mm^3 we reduced again the number of vials until three per cycles. We have included 50 pts with DLBCL and 5 with follicular lymphoma grade IIIb, median age was 61 years (range 34-79), 60% had an high-intermediate or high IPI. CHOP was administered every 14 days, preceded on day 1 by rituximab and followed by 7 (in the first ten patients), 5 or 3 days of G-CSF (filgastrim). Haematological toxicity and feasibility was calculated over 322 cycles administered. We have used 1313 GCS-F vials, 5 vials (range 2-7) for cycle and a median of 25 vials (range 10-35) for every pts. The programmed therapy was completed in 53 out 55 pts (96%); two pts swichted to a different scheduling (R-CHOP21). 11 cycles (3,8%) have been delayed in 9 pts for severe adverse events. Neutropenia grade 3-4 developed in 2.7% of cycles, febrile episodes in 1.2% of cycles, thrombocytopenia grade 3 or 4 in 1.2% of cycles and hospedalization in 1.2% of pts. Of the 322 cycles considered, the median nadir of leucocyte was 3870×10^3/L (range 400-8400), the median nadir of haemoglobin was 11.1 gr/dL (range 5.8-15.5) and the median nadir of platelets was 146000 (range 43000-328000). The complete remission rate was 90% and overall survival was 75% after 3 years. In conclusion in our experience, dose dense chemotherapy (R-CHOP14) supported by G-CSF has been well tolerated by patients, including the elderly. In addition, the clinical outcomes appear to be better with dose-dense than with standard CHOP. The reduction from 10 to 5 GCS-F vials has not determined an increase of neutropenia, febrile episodes, delays in the treatment and hospedalizations confirming and high rate of response to therapy.

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RITUXIMAB-CHOP14 SEEMS TO OVERCOME THE NEGATIVE PROGNOSTIC SIGNIFICANCE OF B CELL ORIGIN IN DLBCL LYMPHOMA PATIENTS

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Diffuse large B cell lymphoma (DLBCL) is the most common types of non-Hodgkin's lympoma. Approximately half of all patients (pts) will be cured of their disease by primary therapy. Two major subgroups were identified by gene expression profiling: germinal centre B (GC) cell or non-germinal centre (non-GC). The GC group showes a significantly better survival than the non-GC group. Immunohistochemistry has been evaluated as a surrogate for this molecular classification. The aim of this study was to define retrospectively the B-cell origin of 40 pts treated with R-CHOP14 and to evaluate if the dose-dense therapy could improve clinical outcome. We performed a centralized immunohistochemical stains on formalin-fixed paraffin-embedded tissues from diagnostic biopsies with the following antibodies: CD10, bcl-6, bcl-2, MUM1 and Mib1. Based on the published algorithm we subdivided the pts in GCB and non-GCB origin. We evaluated also the prognostic value of single protein expression. Twenty-seven pts were male, 22 stage III-IV, 17 presented symptoms at diagnosis 22 showed abnormal LDH value, the IPI was intermediate-high risk or high risk in 13 pts. According to immunohistochemistry analysis 16 pts derived from GC and 24 from non-GC, 12 pts presented a positive CD10, 30 a positivity for bcl6, 19 a positive bcl2 and 27 a positive MUM1. Twenty-nine pts (73%) obtained a complete remission (CR), 8 a partial response (PR) and 3 were non responders (NR). Four out 29 CR pts experienced relapse, three (75%) derived from nonGC. Eight pts died, 4 derived from GC and 4 from non-GC. After a median period of observation of 18 months (range 3-72) the overall survival (OS) was 75% and the failure free survival (FFS) was 57%. The statistical analysis was performed comparing the B cell origin and clinical characteristics, moreover was also evaluated the expression of bcl2 either in GC or in non-GC lymphomas. In univariate analysis normal Beta2 microglobulin and low-intermediate risk IPI were significantly associated with longer overall survival. In univariate and multivariate analysis FFS was significantly higher in low and low-intermediate IPI risk pts. No differences were reported in OS and FFS evaluating the B cell origin. In conclusion we can point out that the intensification could enhance the efficacy of R-CHOP regimen improving the overall survival and FFS in pts with non-GC lymphoma. In this analysis the only significance was the IPI index that affected either OS or FFS.

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SAFETY AND TOLERABILITY OF DEPOCYTE® FOR THE PROPHYLAXIS OR THERAPY OF CNS DISEASE IN HIGH RISK AGGRESSIVE LYMPHOMA

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Central nervous system (CNS) involvement may be associated to systemic non-Hodgkin lymphoma (NHL), and worsens patient prognosis. Intrathecal prophylaxis is suggested for high risk patients, while therapy is indicated in patients with proven CNS involvement. We employed intrathecal lyposomal ARA-C (Depocyte®) as prophylaxis in two patients with high risk ALK-negative anaplastic large cell lymphoma or as therapy in one case of secondary CNS diffuse large B cell lymphoma (DLBCL). The first patient was diagnosed in July 2006. Multiple nodal, as well as pulmonary, hepatic subcutaneous and bone marrow involvement were documented at diagnosis. IPI was 3. The second patient was referred to our institution in August 2006. He complained B symptoms and CT scan revealed multiple subdiaphragmatic adenopathies. Neoplastic involvement of the bone marrow was documented. IPI was 2. The last patient presented with DLBCL involving the connective tissues of the neck, including occipital skull, and widely infiltrating the meninges and cerebellar hemispheres. Laterocervical nodes were also enlarged. Stage was IIE, IPI was 2. The first two patients received induction with megaCEOP (1200 mg/ms cyclophosphamide, 110 mg/ms epirubicine, 1,4 mg/ms vincristine and 40 mg/ms prednisone every 14 days), intensification with MAD (8 mg/ms mithoxantrone, 2 g/ms/12h ARA-C and 4mg/ms dexamethasone, days 1-3), and consolidation with BEAM (300 mg/ms BCNU 1d, 100 mg/ms/12h VP-16 1d, 400 mg/ms ARA-C 3ds and 140 mg/ms melphalan 1d) and peripheral blood stem cell reinfusion. Depocyte® was employed as intrathecal prophylaxis once in every cycle of megaCEOP with no early and/or late toxicity. Both patients are in continuous complete remission at 11 and 13 months, respectively. The third patient showed dramatic subjective and objective response (resolution of XII cranial nerve palsy) during the first 6 days of systemic steroid therapy and after the first Depocyte® administration. Thus, systemic rituximab-megaCEOP chemotherapy was started, and intrarachideal treatment continued. Treatment is ongoing, without any excess of toxicity after 4 Depocyte® administrations. PET performed after the second cycle showed complete disappearance of CNS disease, but persistence of active lymphoma in the neck. Lyposomal ARA-C was administered safely in the cases described. It did not cause any toxicity or chemotherapy cycle delay. The 14 days-schedule significantly reduced procedure-related risks and did not affect patients' quality of life.

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AUTOLOGOUS STEM CELL TRANSPLANTATION FOR PERIPHERAL T-CELL LYMPHOMA, A SINGLE-CENTRE EXPERIENCE

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Background. Peripheral T-cell lymphomas (PTCL) constitute a heterogeneous group of aggressive lymphomas. The prognosis is poor with an overall survival of 25-45% at 5 years in patients treated with conventional dose chemotherapy. Retrospective data shows that patients whose disease is sensitive to conventional chemotherapy may benefit from the use of frontline autologous stem-cell transplantation (ASCT). We present a retrospective series of 11 PTCL patients treated with an upfront (10 patients) or salvage (1 patients) high dose therapy program. Patients and *Methods.* Median age was 58 years (range 27-74). Eight patients (73%) presented with advanced (III-IV) Ann Arbor stage; 8 (73%) had B symptoms; 10 (91%) had high lactate dehydrogenase levels; 2 (18%) had bulky disease; 6 (55%) and 5 (45%) patients presented with ≥3 or ≥2 risk factors according to the International Prognostic Index or the Prognostic Index for peripheral T-cell lymphoma. Induction consisted of dose-dense dose-intense cyclophosphamide, epirubicin, vincristine and prednisone (megaCEOP) in 73% of cases; cyclophosphamide, doxorubicin, vincristin and prednisone (CHOP) in 9%; MACOP-B in 9%; P-VEBEC in 9%. Carmustine, etoposide, ARA-C and melphalan (BEAM) was used as myeloablative chemotherapy in all patients except for one who received mithoxantrone and melphalan as conditioning treatment. *Results.* Nine (82%) patients completed the whole treatment program, while 2 patients did not receive transplant because of insufficient stem cell harvest (1) or unacceptable peripheral and autonomic neuropathy (1). Post-intensification disease status was complete remission (CR) in 4 (36%) patients, CRu in 2 (18%), partial remission in 4 (36%), and refractory disease in 1 (9%). After ASCT 6 of 9 (66%) transplanted patients achieved complete remission, with PR-to-CR conversion observed in 2 cases. With a median follow-up of 37 (range 5-81) months from diagnosis 5 of 11 patients (45%) are alive. Four of these attained CR, either before or after ASCT. Six patients (55%) died as a result of disease progression (3), infectious complications (2), or secondary acute myeloid leukemia (1). *Conclusion.* Disease status at transplant appeared to be the major factor that impacted outcome in our series. Although in a small number of patients, our data suggest that frontline ASCT consolidation may improve treatment outcome for chemosensitive PTCL patients.

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PERIPHERAL CD4+ AND CD8+ LYMPHOCYTES CORRELETES WITH RESPONSE TO RITUXIMAB MONOTHERAPY IN UNTREATED-FOLLICULAR LYMPHOMA PATIENTS

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Rituximab is a chimaeric IgG1 monoclonal antibody, currently indicated for treatment of follicular and aggressive B-cell non-Hodgkin lymphoma patients (pts). It works by binding to CD20, a cell surface molecule expressed on almost all normal and malignant B-cells. Although several immunologic mechanisms have been proposed for Rituximab activity including complement-mediated cytotoxicity (CDC), vaccine like effect, and antibody-dependent cellular cytotoxicity (ADCC), its entirely underlying *in vivo* action is unknown. We hypothesised that the transient B-depletion after Rituximab would result in re-arrangement of the residual immuno-competent lymphocytes and that their absolute count could be predictive of the response to the treatment. Thus, we monitored prospectively the trend of peripheral lymphocyte subsets by flow-cytometry assay prior, during and after Rituximab therapy in naïve-follicular lymphoma (FL) pts. From May 2005 to August 2007 33 untreated FL pts received with 4-weekly infusions of Rituximab at standard dose (375 mg/mq) followed 8 weeks later by restaging of the disease. Nineteen (57%) were female; the median age at diagnosis was 56 years (range: 39-80). Twenty-seven were in advanced stage (stage III-IV) and 8 pts presented bulky-disease; only 3 pts were symptomatic. Thirty pts were evaluable for FLIPI score: 15 belong to the low-risk and 7 to the high-risk category. At 12 weeks from the 1st Rituximab infusion, we obtain an overall response rate (ORR) of 70% (4 complete and 19 partial responses), while 3 pts progressed. In terms of lymphocyte subsets in the responsive pts we observed a statistical significant increase in the absolute count of $CD4^+$ and $CD8^+$ peripheral lymphocytes at the time of restaging versus the baseline values ($p<0.0005$; $p<0.001$), while in the non responders this trend was not described. The NK-cells remained stable all along the observation in both subgroup of pts. These preliminary results suggest a relevant role of non-B lymphocyte subsets related to the anti-CD20 action of Rituximab. This trend showed a significant statistical difference according to the response. Otherwise continuative evaluation in the time could support our observation and define if this non-invasive method could be predictive also of the duration of the response.

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SERUM FREE LIGHT CHAINS RATIO, TOTAL KAPPA AND LAMBDA RATIO AND IMMUNOFIXATION ARE NOT PROGNOSTIC FACTORS IN MULTIPLE MYELOMA

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Introduction. The reduction of the tumour burden by chemotherapy is related to patient survival. Responders survive longer than non responders. However, a controversy is open about the magnitude of the response and particulary on the role of complete response (CR) and its relationship to overall survival (OS). Some authors described a longer survival for patients reaching CR, where others were not able to find this advantage. We investigated, in a retrospective analysis, if there is a predictive role of sFLC ratio, total kappa and lambda ratio (KLR) and serum IFE, in a cohort of MM patients who underwent autologous stem cell transpantation (SCT) or tandem autologous and allogenic SCT. *Patients and methods.* Serum IFE, KLR and sFLC ratio were performed in 203 MM pts, 3 months (range 1-5 months) after autologous (173 pts) or tandem autologous and allogenic SCT (30 pts): 154 pts had MM secreting intact immunoglobulin paraproteins (IIMM), 47 pts had light chain MM (LCMM), 2 pts were non secretory MM (NSMM). Range values for KLR were 1.1-2.9, and 0.26-1.65 for sFLC ratio. OS and event free survival (EFS) were evaluated with Kaplan-Meier statistical method. *Results*. Fifty-one/203 (25%) pts were negative by serum IFE, 99/203 (49%) pts reached a normal KLR and 102/203 (50%) pts presented a normal sFLC ratio. The median duration of OS was 54.3 months (95% CI: 45-64.7 months) and median EFS was 19.5 months (95% CI: 16.3-22.6 months). OS was not significant for all three parameters: KLR (p=0.10), sFLC ratio (p=0.83), IFE (p=0.81). Event free survival (EFS) was near significantly for serum IFE (p=0.07), significant for KLR (p=0.016) and not significant for sFLC ratio (p=0.24). *Conclusion.* The statistical analysis has shown that all these parameters evaluated 3 months after autologous or tandem autologous/allogenic SCT are not predictive of a longer overall survival. Probably, reaching a normal KLR without CR by IFE or sFLC ratio is suitable for a stable disease characterized by a later incidence of relapse. So, sFLC are a prognostic factor at diagnosis in all plasma cell disorders but not if used to evaluate the quality of the response after autologous SCT.

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CXCR4 AND CD43 EXPRESSION IN PLASMA CELL MALIGNANCIES

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Bone marrow (BM) microenvironment has been envised as a "trap" for circulating multiple myeloma (MM) precursors, which expand and differentiate to malignant plasma cells within the BM, invading the peripheral blood only in the terminal stage of the disease, i.e. plasma cell leukemia (PCL). While several adhesion molecules have been identified that promote the binding of malignant plasma cells to BM accessory cells and extracellular matrix (i.e. CXCR4), little is known on molecules able to prevent such interactions, by eventually favoring the escape of tumor cells from the BM "trap". Previous studies have indicated that the CD43 sialoglycoprotein functions as an anti-adhesion molecule by providing a repulsive barrier around cells due to its extended conformation and negative charge of sialylated residues. We have analyzed the bone marrow blood of 40 patients with MM and 8 with PCL. 26 out of 40 MM patients presented a IgG component, while the remaining 14 were IgA. On the basis of the staging criteria, 23/40 patients were in stage I-II and 17/40 in stage III. We evaluated CXCR4 and CD43 expression by flow cytometry. Mean fluorescence intensity ratios (MFIRs) were calculated by dividing the mean fluorescence intensity for CXCR4 and CD43 by the mean fluorescence of the respective nonspecific isotype control. 23/40 MM patients (stage I-II) showed high CXCR4 expression (median MFIR 15.2; range: 10.3-50.4) while 17/40 (stage III) showed lower CXCR4 expression (median MFIR 6.5; range: 5.1-9.5). All MM patients were CD43 negative. By contrast all PCL patients were CD43 positive (MFIR 7.8; range: 6.2-10.1), while CXCR4 expression was like MM stage III patients group (MFIR 6.2; range: 4.8-8.9). Our results that malignant plasma cells from MM lack surface CD43 as opposed to PCL in which CD43 expression was found at a high cellular density, indicate that CD43 expression may be related to the biological and clinical progression of MM. Moreover the possible prognostic role of CXCR4 in MM warrants further clinical investigation on a larger series of patients even on the basis of future therapeutic strategies.

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INTEGRATIVE GENOMIC APPROACH IDENTIFIES DEREGULATED MICRORNAS IN HUMAN MYELOMA CELL LINES

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The role of microRNAs (miRNAs) in multiple myeloma (MM) remains to be fully elucidated. To identify miRNAs potentially deregulated in MM, we performed an integrative analysis of genome-wide DNA copy number (CN), gene expression profiling and miRNAs expression profiling in a panel of 16 human myeloma cell lines (HMCLs). Global miRNA and mRNA expression data were generated on Agilent miRNA microarrays (representing 470 human mature miRNAs) and Affymetrix GeneChip HG-U133A arrays, respectively; genome-wide profiling data were generated on Affymetrix GeneChip Human Mapping 250K Nsp arrays. CN values were inferred using the DNAcopy R Bioconductor package. To measure the correlation between the expression levels of each miRNA and the corresponding CN values, non-parametric analyses were performed (Kendall's tau correlation). We identified 22 miRNAs with a good correlation ($p<5\times10^{-2}$) between expression levels and local CN variations, suggesting that a dosage effect could be a main mechanism underlying their expression. The identified miRNAs are mapped within different genomic regions, including chromosome 22q11.21, 8q24.22, 17, 7 and 16q22, and for some of them a role in other types of cancer as well as in MM has already been reported or suggested. The same approach was applied in order to investigate the association between intronic miRNA and corresponding host-gene expressions. A positive correlation ($p<5\times10^{-2}$) was found for 22 intronic miRNAs mapped within 20 different deregulated host genes, strongly supporting the suggestion that intronic miRNAs and their host genes share the same regulatory sequences and can be co-transcribed. Among the most correlated miRNA/host-gene pairs we identified miR-342-3p/EVL and miR-335/MEST, for which the miRNA expression levels were validated by means of Q-RT-PCR. The presence of a correlation was further confirmed in a fraction of primary tumors. Some miRNA/host-genes expression also shared a significant correlation with CN variations. In conclusion, we demonstrated that the presence of genomic lesions and the expression of host-genes can affect miRNA expression in HMCLs. We are now attempting to investigate these findings in primary tumors in order to better define the role of miRNAs in the pathogenesis of multiple myeloma.

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A NEW NON THROMBOGENIC SCHEDULE FOR *IN VIVO* PURGING IN PATIENTS WITH MULTIPLE MYELOMA: PRELIMINARY RESULTS OF A PILOT STUDY

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In the past decade, thalidomide, bortezomib, and lenalidomide have emerged as highly active agents in the treatment of MM. However, the optimal schedule and association of these drugs are still under investigation. Thalidomide has shown to be highly thrombogenic in induction therapy. On the contrary Bortezomib is not and has very rapid antimyeloma activity; peripheral blood stem cell (PBSC) collection is feasible after therapy with bortezomib. *In vitro* data suggest synergistic effects when bortezomib is combined with some alkilators and Cyclophosphamide

(CY) is effective in treatment of MM, but not stem cell toxic. We started a pilot study in high risk newly diagnosed MM patients, combining bortezomib, CY and dexamethasone (DEX) as induction and mobilizing therapy to assess: feasibility, percentage of complete remission (CR) and near CR (nCR) before (primary end point) and after stem cell transplantation (SCT), percentage of PCR negativity and monoclonal plasmacells in PBSC harvest and in patients at day +90 after SCT. Patients receive three 3-week treatment cycles with bortezomib 1.3 milligrams/squared-meter on days 1, 4, 8, and 11 in combination with DEX 40 milligrams/day i.v. on days 1,4,8,11 and CY i.v. 300 milligrams/squared-meter on days 1,8,15. After third course, patients undergo stem cell mobilization with bortezomib 1.3 milligrams/squared-meter on days 1,4,8,11 in combination with DEX 40 milligrams/day i.v. on days 1,4,8,11 and CY i.v. 3 grams/squared-meter on day 8 followed by G-CSF until leukaphereses. Patients who succeed to mobilize an adequate number of PBSC were planned to receive conditioning therapy according to this schedule: bortezomib 1.3 milligrams/squared-meter on days -8 and -4 plus Melphalan 200 milligrams/squared-meter on day -1. At present 6 patients have been enrolled (Table 1). In 3 we were able to collect PBSC and 3 underwent SCT. With a median follow up of 96 days all patients are alive. The hematological toxicity was negligible and we did not observe neither thromboembolic events nor grade 3-4 neurotoxicity. This preliminary experience shows that this schedule is well tolerated also in elderly patients with a very high risk rate. We need to further increase the number of patients and to have a longer follow up in order to assess the impact of this approach on response rate and PFS in these patients.

Table 1. Baseline characteristics of patients and response.

Patients	Age	Sex	Stage	ISS stage	Previous lines of treatment	PBSC harvest/ ASCT performed	Response before ASCT	Outcome
1	59	F	III A	III	1	yes/yes	nCR	alive
2	68	M	III B	III	2	yes/yes	nCR	alive
3	73	F	III A	III	0	no/no	n.e.	alive
4	72	F	III A	III	1	yes/yes	VGPR	alive
5	69	M	II A	I	0	no/no	n.e.	alive
6	77	F	III A	II	1	no/no	n.e.	alive

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MOBILIZATION OF PERIPHERAL STEM CELLS WITH HD CYTARABINE IN MULTIPLE MYELOMA PATIENTS: A SINGLE CENTRE EXPERIENCE

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High dose chemotherapy with HD Ara-C was primary designed for collection of peripheral blood stem cell (PBSC) in lymphoma patients, at risk of low mobilitation, candidates for autologous stem cell transplantation. From 2002 to 2007, in our centre, 12 multiple myeloma patients were mobilizated with Ara-C 2 gr/sm twice a day, for 3 consecutive days, plus G-CSF 10 micrograms/ Kg/day. Time to collection was fixed by a count of CD34 circulating >15 cells/microliter (median day to collection +11; range 9-21). All large volume leukapheresis procedures were performed with As.Tec204 or Com.Tec Fresenius, flow continuous blood separator, mononucleated program, P1Y. The median number of apheresis for each patient was 1,6 (range 1-4) with a median number of CD34 cells harvested of $10,4\times10^6$ (range 2,7-30,1), with a median peak of 70 (range 15-180) CD34 cells/microliter at time of yeld. The extra haematologic toxicities were moderate (no toxicity grade 4) and median duration of neutropenia (<500) and trombocytopenia (<50000) was 10 (9-12) and 12 days (11-15), respectively. Only one patient failed mobilization, while 2 patients who previously failed mobilization with HD CY, were successful harvested. Nine patients were admitted at autologous transplantation planning. Tandem transplantation was performed in 6 patients. Condictioning regimen, in all patients, was HD Melphalan (200 and 140 miligrams/m^2 in tandem autoPBSCT). Complete and durable engraftment was obtained in 7 patients, with a prompt hemopoietic recovery. Two late engraftments were observed. In every case, no related transplant mortality was observed. Thus, HD Ara-C is an highly effective mobilitation scheme in multiple myeloma patients, allowing good CD34 cells yeld, also in "poor mobilizer" patient.

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IMMUNOPHENOTYPIC ANALYSIS COMBINED WITH POLYMERASE CHAIN REACTION ASSAY OF IGH GENE FOR THE DIAGNOSIS OF PLASMA CELL DISORDERS

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Background. Plasma cell disorders include malignant conditions (Multiple Myeloma, MM) and benign or premalignant disorders (Monoclonal Gammopathy of Undetermined Significance, MGUS). Moreover, two clinical variants, smoldering (sMM) in which the diagnosis criteria for myeloma are met but the disease may be stable for long period and the patients are asymptomatic, and that requiring treatment (crabMM). The phenotype of myeloma plasma cell (PC) has long been considered the same as that of normal PCs. Only recently, the differential expression of surface markers on normal versus malignant PCs has been described. While neoplastic PC retain some of the phenotypic characteristics of normal PC, such as strong CD38 expression, immunophenotyping studies demonstrated that myelomatous PC lack CD19 and may express CD56. However, the present-day diagnostic criteria are not completely satisfactory as a subset of individuals are not correctly categorized. This differentiation is particularly difficult in patients with borderline criteria between MGUS and clinically indolent (sMM) or active (crabMM) myeloma. *Aim*. The rationale of this study was to detect the incidence of the malignant bone marrow (BM) PC phenotype in MGUS compared to MM. Moreover, we addressed the issue of whether a different PC phenotype distinguished sMM from crabMM cases. Finally, the possible utility of immunophenotypic analysis combined with PCR assay was explored to detect the incidence of malignant clone among the different plasma cell disorders. *Methods.* PCs were identified by their characteristic light scatter distribution and by a sequential gating strategy, assessing CD19, CD45, CD38 and CD56 reactivity by using an EPICS Profile II flow cytometer (Coulter Electronics, Inc., Hiealeah, FL). DNA from 80 patients was extracted from whole blood and analyzed for clonal IgH gene rearrangement by polymerase chain reaction (PCR) using two different combinations of VH FR3 and JH primers. PC population was estimated by PCR capillary electrophoresis on a 3130 Genetic Anayzer . We combined the two methods for the final analysis. *Results*. Fifthy-one MM (19 sMM and 32 crabMM) and 29 MGUS entered this study. A statistically significantly higher percentage ($p<0.0001$) of $CD19^+$ BMPC were demonstrated in MGUS (33+2 s.d.) as compared with sMM (5+5 s.d.) and crabMM (2+3 s.d.). In contrast, a progressive increase of $CD56^+$ PCs was found from MGUS to crabMM through an intermediate value observed in the sMM group (MGUS 25+32 versus sMM 61+40 versus crabMM, 69+37, $p<0.0001$). Using 30% as cut-off value for both immunophenotypic profiles, a low CD19 expression correctly clustered all sMM and crabMM cases (CD19low), while 14 out of the 29 MGUS cases (48.3%) showed high CD19 expression in the BMPC. On the other hand, high CD56 expression, predicted 75% (24/32) of crabMM, 74% (14/19) of sMM, and only 8/29 (17%) of MGUS group ($p<0.0001$). Moreover, we were able to split cases in 3 groups with a different PC phenotype, namely neoplastic PCs ($CD19^{low}/CD56^{high}$), normal PC ($CD19^{high}/CD56^{low}$) and PC with an intermediate phenotype. The neoplastic clone identified by flow cytometry was detected in 6/29 (21%), 14/19 (74%) and 24/32 (75%) of MGUS, sMM and crabMM, respectively. In order to better dissect among PC disorders, BMPC clones were also detected by using PCR based assay, resulting polyclonal pattern in 25 cases, oligoclonal pattern in 23 cases and monoclonal peak in 32 cases. A statistically significant distribution was observed between the CD56 expression and PCR results. In particular, among cases displaying CD56>30%, 25/32 (78.1%) showed a monoclonal pattern, while 10/23 (43.5%) and 11/25 (44%) cases were oligoclonal and polyclonal, respectively. On the other hand, no statistically association was found between PCR and CD19 expression results. Finally, PCR analysis displayed a monoclonal pattern in 16/32 crabMM, 8/19 sMM and 8/29 MGUS cases. *Conclusion*. Our results indicate that flow cytometry analysis may clearly identify neoplastic BMPC, with, as expected, the worst phenotype more prominent in MM cases. PCR analysis failed to give additional information over flow cytometry in predicting accurately the diagnosis. Whether the presence of both the high expression of CD56 and the monoclonal PCR pattern in MGUS cases may have an impact in predicting a different clinical outcome should be evaluated.

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NO SPONTANEOUS OSTEOCLAST FORMATION IN AN *IN VITRO* MODEL DERIVED FROM PRIMARY PLASMA CELL LEUKEMIA

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We developed an *in vitro* model of spontaneous osteoclastogenesis, derived from peripheral blood mononuclear cells (PBMCs) of patients with multiple myeloma (MM)-lytic bone disease. In those patients, by means of this model, we previously showed that T cells support the formation of osteoclasts (OCs) with longer survival (BLOOD 2004;104: 3722). The spontaneous osteoclastogenesis, detected in the unfractionated PBMC cultures from patients with MM-lytic bone disease, was instead absent in the parallel T-cell-depleted cultures as well as in the unfractionated or T-cell depleted cultures from MM patients without lytic bone disease. Moreover, in these previously negative cultures, OCs not exhibiting a longer survival developed following the addition of recombinant human macrophage-colony stimulating factor (rhM-CSF) and receptor activator of nuclear factor-kB ligand (rhRANKL). Our purpose was to use this *in vitro* osteoclastogenesis model in primary plasma cell leukaemia (pPCL) as well. After obtaining patient's informed consent, PB was collected from 2 women (57- and 58-years-old, respectively) diagnosed as having pPCL, according to IMWG criteria. One of them had 2 small lytic lesions in her skull and 1 in a rib, whereas the skeleton standard radiography was negative in the other. MCs were isolated by Ficoll-Hypaque density gradient centrifugation, and T-cell-depletion was performed by using anti-CD2 antibody-coated immunomagnetic Dynabeads. Unfractionated or T-cell-depleted PBMCs were cultured for about 30 days in the appropriate conditions, and in the presence or absence of rhM-CSF and rhRANKL, as described elsewhere. At the end of the culture period, no OCs developed in both the culture-types. In them, OCs not exhibiting a longer survival were however detected after the addition of the recombinant cytokines. The controls were included in our previous study. These results, demonstrating that rhM-CSF and rhRANKL were necessary to trigger and sustain osteoclastogenesis in the cultures from the patients with pPCL, overlapped those yielded from MM patients without lytic bone disease or other controls. In conclusion, the use of this *in vitro* model did not show a T-cell support of osteoclastogenesis in pPCL. These findings may contribute to the study of this very rare disease, with a poorly understood biology.

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CYCLOPHOSPHAMIDE, ADRYAMICIN AND DEXAMETASONE IS HIGHLY EFFECTIVE THERAPY IN NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS: A SINGLE CENTRE EXPERIENCE

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In the era of novel drugs in multiple myeloma, VAD-like chemotherapy regimen is still the gold standard of induction treatment in patients elegible to autologous peripheral blood stem cell transplantation (autoPBSCT). From 2005 to 2007, in our centre, we have treated 10 newly diagnosed myeloma patients, candidates to autoPBSCT, with 2-3 cycles, every four weeks, of CAD protocol (Cyclophosphamide 1500 miligram/m^2 days 2,3; Adriamicyne 40 miligram/m^2 days 1,2; Dexamethasone 40 miligrams days 1-4). Haematologic toxicity grade 3 and 4 was observed in all patients, gastointestinal grade 3-4 in 5/10, infectious grade 3-4 in 3/10 and dermatologic grade 3-4 in 1/10 patients. Eight/10 patients obteined a partial remission (1 VGPR, 1 PR>75% 6 PR >50%) and 2/10 had no change. No patient underwent progression disease during treatment and no mortality was observed during this induction phase. Despite of impressive overall response rate (80%), no RC was observed and virtually all patients experimented one or more toxicities grade 3-4. In our experience, CAD seems to be is a highly effective chemotherapy regimen in newly diagnosed multiple myeloma patients, expecially if extensive chemotherapy debulking is needed prior PBSC collection. However, definitive results can't be drawn, because of the small number of patients treated.

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ASSESSMENT OF PROGNOSIS IN MULTIPLE MIELOMA PATIENTS TREATED WITH BORTEZOMIB-DEXAMETASON BASED THERAPY: PROPOSAL OF A SCORING SYSTEM. A MONOCENTRIC STUDY

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Background. Therapy of multiple mieloma (MM) with new agents as velcade has frustrated prognostic value of classic prognostic factors as deletion of chromosome 13 or disease stadium. Actually ther' are not prognostic factors that clearly allow to define treatment response to therapies based on velcade and dexametason combination. *Aims.* Aim of this study is to define which elements have prognostic value in patients treated with therapies based on velcade-dexametason combination. *Methods.* This is a retrospective study. All patients were treated with velcade-dexametason based therapy, with or without thalidomide or doxyl administration. We considered the following factors as risk factors: age, sex, Salmon and Durie stage, renal failure, monoclonal component isotype, number of velcade-dexametason based cycle administred, number of previous treatments, lymphocyte count below 1000/mcl and platelets count below 50000/mcl and CMV and HZV infection at second therapy cycle (the majority of patients showed lymphocyte and platelets nadir and CMV and HZV infections at second treatment cycle). *Results.* Median number of therapy cycles administered was 4 (R 2-9). M/F ratio was 10/9. Median age was 68 years (R 49-81). All patients showed advanced disease (Salmon and Durie stage III). 4/19 (21%) patients had renal failure. 9/19 patients (47%) showed a monoclonal component IgG/k or lambda type, 3/19(15%) IgA/K and IgA/l type, 2/19(10%) IgD and micromolecular type. 3/19 (15%) patients presented del of cr 13. 8/19 patients (42%) were in first line therapy; 11/19 (57%) were in third and in fourth lines therapy. Their median follow up was 25 mounths (7-43). After treatment 8 patients were in progression disease (PD), 1 in stable disease (SD), 7 in complete remission (CR) and 3 in very good partial remission (VGPR). At univariate analysis only the second or higher line of therapy, lymphocyte count below 1000/mcl and platelets count below 50000/mcl and CMV or HZV infection at second therapy cycle showed an association trend with response to treatment. We attributed one point to each of these variables. All patients in CR and VGPR showed a score of 0-2 (10 patients), while patients in SD or PD showed a score of 0-2 (4 patients) or 3-4 (5 patients), with a Chi Square test of 0.02,a Fisher's text of 0.011 with a RR of 3.5 and an indeterminated OR and a positive predictive value of 1 and a negative predictive value of 0.7. *Conclusions.* In MM patients treated with a velcade-dexametason based therapy, with or without thalidomide or doxyl, the presence of 0 or 2 of these factors: line of therapy, lymphocyte count below 1000/mcl and platelets count below 50000/mcl infection at second therapy cycle and CMV or HZV infection at second therapy cycle is useful to define patients at good prognosis and responsive to treatment.

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SAFETY OF LENALIDOMIDE IN HEMATOLOGICAL MALIGNANCIES :A REVIEW OF 27 CASES

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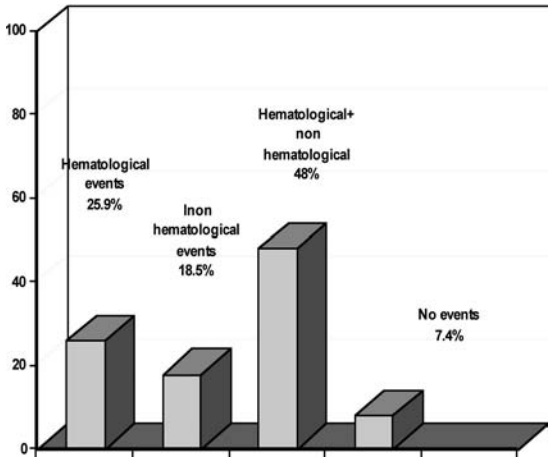
Lenalidomide, a promising IMiD, has clinical activity in several hematological malignancies. Our aim was to evaluate the safety profile, by examining our experience with this new drug. The characteristics of the patients (pts) are summarized in Table 1. Hematological events: grade 2-3 neutropenia occurred in 63% of pts, but it did not require any therapy with G-CSF and it was not associated with fever. The only case of grade 4 neutropenia, delayed the beginning of the next chemotherapy cycle by 4 weeks. In 18.5% of the cases, a grade 3 thrombocytopenia occurred, however it did not require any transfusion and it was not associated with bleeding disorders.In pts that showed anemia(11.1%) at the onset a progressive improvement was reached in relation to the entity of the response. Extra- hematological events: in 19% of pts, constipation of grade 2-3 occurred, but only 1 case required a dosage reduction from 10

mg/day to 2,5 mg/day for 21 days. Occasional constitutional symptoms occurred and, there was only a case of grade 1 skin toxicity. The infective events (1 Herpes Zoster, 1 Aspergillus Fumigatus pneumonia, 2 broncopneumonia of unknown etiology, 1 episode of fever and cough) were 18%. 7.4% of the pts have shown vascular events: a retinic venous thrombosis, resolved after therapy with LMW heparin, and an episode of ischemic colitis. In these 2 pts thrombophilic screening was negative, and also the doppler of abdominal vessels of the patient with ischemic colitis did not show any occlusion. In this last case, after a gastroenterological consulting, we decided to suspend lenalidomide. During the treatment, 2 pts died: one patient, already affected by heart disease, died of heart failure, and the other one died of pneumonia. Both pts, affected by MM advanced and refractory with a non favourable PS and previously treated with several therapeutic strategies, died, respectively, after 2 and 1 cycle of treatment.Our experience corresponds with the data suggested by literature. The hematological events have been easily managed, without requiring any supportive therapy. The pts, always, need an antithrombotic prophylaxis, and ongoing studies will decide on the best one. Prophylaxis with broad-spectrum bacterial antibiotics and antifungal agents is necessary to prevent potentially severe infective events particularly in pts previously treated and with a non favourable PS. In conclusion, lenalidomide is safe and well tolerated in patients with hematological malignancies.

Table 1.

Total	27
Median age	67 (range 48-78 years)
M/F	16M/11F
Diagnosis (from 2005)	25 MM, 2 5q-Syndrome
Previous treatment	No : 14 (52%) Yes : 13 (48%)
Single agent	2 pts (10 mg/day for 21 days every 4 weeks)
Association	Alkylating agents (10 mg/day for 21 days every 4 weeks):21pts Dexamethasone(25 mg/day for 21 days every 4 weeks):4 pts



P182

PRIMARY PLASMACYTOMA OF PANCREAS AND LIVER: A RARE EXTRAMEDULLARY LOCALIZATION OF PLASMACYTOMA SIMULATING CARCINOMA OF THE HEAD OF THE PANCREAS WITH LIVER METASTASES

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Introduction. Extramedullary plasmacytoma is a rare variant of plasma cell tumor involving organs outside the bone marrow. The vast majority of extramedullary plasmacytomas present as a secondary tumor of systemic myelomatosis of the bone marrow. We experienced a patient with extramedullary plasmacytomas of the pancreas and liver simulating a tumor of pancreas with liver metastases. *Case report.* A 81 year-old man was submitted for dyspepsia to hepatic ultrasound and to computed tomographic scans that revealed a homogeneous mass in the head of pancreas of 2.5 cm of diameter and three solid focal hepatic lesions of diameter mm.17 and mm.31 compatible with metastatic lesions. It was submitted to a biopsy of liver that showed a histological picture of hepatic metastases by differentiated carcinoma like pancreatic origin. At immunohistochemistry: hepatocyte, AFP, cromogranina, NSE, sinaptofisina, CA19-9, CK7 and CK20 were negative and CEA had a focal positivity. The bone scintigraphy didn't showed bone secondary lesions. The patient began treatment with Gemcitabina at dose of 1000 mg/m^2 at days 1, 8, 15, 22 and after the first cycle a new ultrasound examination verified a increase of number and dimension of the liver lesions. The case was hisologically reconsidered and were also tested the CD 138 and the citocheratine that were intensely positive. The final haematological diagnosis was extramedullary plasmoblastic myeloma of the pancreas and liver. Bone marrow biopsy was negative; negative resulted also the skeletal radiological examinations and there weren't monoclonal component. The patient started chemotherapy with liposomial doxorubicna (mg 20/m^2 at days 1,15) plus bortezomib (mg 1.3 /m^2 at days 1, 8, 15, 22) plus desametasone (20 mg at day 1, 2, 8, 9, 15, 16, 22, 23). Just after the end of first cycle of chemotherapy ultrasound examination showed a stable lesions on the liver but with a variation of ecografic pattern with central necrosis. The general condition of the patient has improved and the chemotherapy was good tolerated. *Discussion.* Plasmacytomas occurring in extramedullary sites are rare tumours, particularly so when located in the gastrointestinal tract. The plasmoblastic myeloma of liver and the pancreas is a exceptionally entity and in literature are not reported similar cases. The initial response to chemotherapy ago hopes for a good result at the end of the eight programmated cycles.

P183

BCL-1 POSITIVE WALDENSTRÖM DISEASE: CASE REPORT

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Waldenström's macroglobulinemia (WM) is a type of plasma cell neoplasm or B-cell lymphoma characterized by hyperviscosity, or thickening, of the blood. In these kind of lymphomas, certain plasma cells become abnormal, or cancerous, and begin to grow uncontrollably, overproducing large amounts of identical (monoclonal) IgM antibody. This IgM also is called M protein, for monoclonal or myeloma protein. It is classified as a low-grade or indolent form of lymphoma because it is a slow-growing cancer that produces fewer symptoms than other types of lymphomas. and often does not require treatment. To date, chromosomal abnormalities specific to the disease haven't been recognized, even if it's reported that the frequency of partial deletion of the long arm of chromosome 6 and trisomy 4 distinguishes WM from other chronic malignant B-cell proliferations. We report a case of 78 years old man with lymphoplasmacytic lymphoma/Waldenström macroglobulinemia, as diagnosed by the presence of a serum monoclonal IgM component of 3g/dL and a positive lymphonodal biopsy for this pathology (CD19$^+$, CD20$^+$, CD38$^+$, CD5$^-$, CD10$^-$, ciclin D1$^-$, SmIgK$^+$). Interestingly, the analysis by real time PCR of DNA from bone marrow of the patient showed the bcl-1 gene over expression. At diagnosis the patient presented marked splenomegaly, high LDH serum level (780 UI/L) and mild anemia (Hb 10.8 g/dL). Total body TC scan showed lymph node enlargement in each site observed. Clinical outcome was very poor: the patient didn't response to the therapy and died three months after the diagnosis. At our knowledge, this is the first BCL-1 positive Waldenström case reported. The aggressive clinical outcome of the patient warrant to investigate bone marrow bcl-1 gene expression in all lymphoproliferative diseases, including Waldenström macroglobulinemia, in order to identify, at the diagnosis, the subset of patients whose prognosis could be worst.

Myeloma and Gammopathies (II)

P184

P16/INK4A GENE METHYLATION STUDY IN MULTIPLE MYELOMA AND MGUS BY MS-PCR

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Methylation of gene promoters is an important mechanism of inactivation, together with deletion and mutation, of tumour suppressor genes. In the last years, it has been described high methylation rates of p16/INK4a gene, wich is involved in cell cycle control, in monoclonal gammopathies (MG) and particularly in Multiple Myeloma (MM) bone marrow cells (BMC). Different methods can be used to assess the methylation status of a gene and can be summarized in 3 groups: 1) use of restriction enzymes that have different activity upon the methylation state of the substrate DNA, 2) Chromatin immunoprecipitation with proteins that have different affinity upon the methylation state of the substrate DNA and 3) MS-PCR, a chemical method which deaminates the unmethylated cytosines to uracile using sodium bisolphite, followed by two alternative PCR reactions specific for the DNA sequence either containing cytosines or uraciles. For this study we used the MS-PCR method (Herman *et al.*, 1998) because it is easy to standardize and it is independent from availability of restriction sites or affinity substrates. *Patients and methods*. We assessed the p16 methylation status in 47 MM and 15 MGUS patients. MGUS is considered to be a pre-neoplastic disease that can evolve to MM by subsequent tumorigenic events that increase genetic abnormalities. BMC genomic DNAs were modified with sodium bisulphite and amplified with PCR specific for both methylated (p16MET) and unmethylated (p16UNM) sequences of p16 gene promoter. In 3 MM patients previously assessed as p16MET$^+$, BM cells were sorted for CD138$^+$ and CD138$^-$ populations. *Results.* 1) p16MET$^+$ is specific for neoplastic plasma cells (PC): on sorted CD138$^+$ and CD138- cells of the same BM only CD138$^+$ were p16MET$^+$, while CD138- cells were p16UNM$^+$. 2) MS-PCR is a sensitive method: we were able to detect a p16MET$^+$ PCR band even in patients that had less than 0.5% of PC in the BMC. 3) p16MET$^+$ distribution is significally different among MM and MGUS patients: we found p16MET+ samples in 38/47 (79%) of the MM patients and only in 5/15 (33%) of the MGUS patients (p=0.0018). *Conclusions.* MS-PCR is a sensitive and specific method to assess p16 methylation in MG. Due to its sensitivity, p16 methylation may be used as a marker in minimal residual disease detection for p16MET$^+$ patients. A lower number of p16MET$^+$ MGUS patients compared to MM patients, suggests that p16 methylation might be one of the mechanisms involved in the transition from MGUS to MM.

P185

SIMULTANEOUS INHIBITION OF THE RAF/MEK/ERK AND BCL-2 PATHWAYS RESULTS IN STRIKINGLY SYNERGISTIC INDUCTION OF APOPTOSIS IN MULTIPLE MYELOMA CELLS

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We have recently obtained evidence that ABT-737, a Bcl-2/Bcl-xL inhibitor, shows potent *in vitro* growth-inhibitory and pro-apoptotic activity on multiple myeloma (MM) cell lines and on primary CD138$^+$ from MM patients. Cross-talk between the Raf/MEK/ERK pathway and the Bcl-2 family has been already reported (Cancer Cell 10, 1-14, 2006). Since we have demonstrated that selective MEK inhibitors (MEK-I), i.e. PD98059, CI-1040, etc., potently inhibit the growth of acute leukemia (AL) cell lines and *ex vivo* cultured primary AL blasts, here, we aimed at analyzing the impact of the simultaneous inhibition of these two pathways on cell growth inhibition and on apoptosis induction in MM cells. We exposed different cell lines (KMS18, KMS27, ARH-77) to increasing concentrations of MEK inhibitors (MEK-I) and ABT-737 (10-1000 nM) (kindly provided by Abbott Laboratories), alone and in combination. While single compounds inhibited cell growth in a dose-dependent fashion, we observed that their combination dramatically enhanced this effect. Similarly, mitochondrial membrane depolarization was greatly promoted when the small-molecules were used in combination. We then analyzed the effects on apoptosis induction. MEK-I induced minimal apoptosis, as determined by sub-G1 peak (13.13±2.6% after 48 hours of exposure to 1000 nM in KMS18 cells). Conversely, ABT-737 as a single agent dose-dependently induced apoptosis (6.7±0.5%, 11.8±3.1%, and 83.7±4.0% at 10, 100, and 1000 nM, respectively). When the two inhibitors were used in combination, more than 50% of the cells became apoptotic at 100 nM of each inhibitor. The quality of the interaction, as defined using isobologram analysis, revealed a remarkable synergism of action with a combination index (CI) of 0.2 at 100 nM of each inhibitor. Similar results were obtained in two other MM cell lines tested, although ARH-77 proved less susceptible to combination-induced apoptosis. Western blot analysis is ongoing in order to identify relevant downstream targets of MEK-I and ABT-737. In conclusion, we demonstrated a striking synergistic pro-apoptotic activity of the combined inhibition of Raf/MEK/ERK and Bcl-2 signaling in MM cell lines. We propose that the simultaneous disruption of these two pathways may lower the apoptotic threshold and deregulate multiple pro-survival pathways in malignant plasma cells as well as in the microenvironment compartment, thereby suggesting potential therapeutic applications of the combined MEK/Bcl-2 inhibition strategies.

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INTEGRATIVE GENOMICS BASED ON MICROARRAYS ANALYSES REVEALS A CORRELATION BETWEEN ALLELIC IMBALANCES AND GENE EXPRESSION PATTERNS IN MULTIPLE MYELOMA

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Multiple myeloma (MM) is characterized by marked genomic instability. Beyond structural rearrangements, such as the well-characterized 14q32 translocations, a relevant role in the biology of plasma cell neoplasia is represented by allelic imbalances that lead to a significant variation in the ploidy status. To provide insights into the genomic complexity of plasma cell neoplasms, we analyzed a representative and stratified panel of MM patients combining fluorescence in situ hybridization (FISH) and microarray high-throughput approaches. Genome-wide profiling data of 40 MM, 4 plasma cell leukemia and 1 monoclonal gammopathy of undetermined significance patients were generated on GeneChip Human Mapping 50K Xba arrays. Copy number (CN) values were inferred using DNAcopy R Bioconductor package. Based on available FISH information, CNs were scaled to assign the values corresponding to exactly two alleles to a nominal multiplicity of 2. A k-means clustering algorithm was used on the cumulative profile of all the data to determine the thresholds for inferring discrete CN values. Hierarchical clustering analysis was applied to identify the natural grouping of genome profiles, and showed that chromosome 1 alterations, chromosome 13 and 14 deletions and hyperdiploidy are the main genetic aberrations driving samples grouping; noteworthy, patients with aneuploid genotypes represent a well-defined cluster. This evidence was also confirmed applying a non-negative matrix factorization clustering procedure. We identified high frequencies of allelic losses at regions 4p, 6q, 8p and 16q that warrant further investigations. Mapping information was integrated with the gene expression profiles of the tumor samples generated on the GeneChip HG-U133A arrays. A multiclass analysis of the identified clusters showed mainly the prevalence of transcripts characterizing the hyperdiploid samples, whereas a non-parametric analysis revealed a strong correlation between normalized gene expression levels and local CN variations (Kendall's tau correlation, 1341 probes at a q-value<0.05, the most significant of which mapped to 1q). This indicate a wide gene-dosage effect suggesting that genomic structural abnormalities in multiple myeloma closely reflect in expression imbalances. Our data reinforce the importance of using novel high-throughput approaches to provide insights into the characterization of novel genetic lesion in primary myeloma tumors.

P187

LACK OF ACTIVITY OF LENALIDOMIDE ON OSTEOBLASTS

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Lenalidomide (LE) is a new promising drug for treatment of Multiple Myeloma (MM). Its activity has been demonstrated by several clinical studies but its mechanism of action is only partially known. However, besides a direct or indirect effect of lenalidomide on MM plasmacells, it has been demonstrated that Lenalidomide is able to inhibit *in vitro* osteoclast precursors although it is not known if this property is maintained *in vivo*. To further explore the effect of Lenalidomide on the physiopathology of the bone disease in MM, we evaluated the possible effects of this drug on osteoblatic differentiation of Mesenchymal Stem Cells derived from bone marrow (BMSCs). BMSCs are multi-potent non-haematopoietic progenitor cells that differentiate into osteoblasts, adipocytes, chondrocytes, skeletal myocytes and nervous cells. Culture of normal human BMSCs were treated with osteogenic medium (OM) with or without Lenalidomide 10 µM. By RT-PCR, we assessed mRNA expression of osteogenic markers such as RUNX2, osteocalcin (OCN) and bone morphogenetic protein (BMP-2) at 21 days of culture. Lenalidomide alone does not increase expression of these markers respect to BMSCs cultured in standard medium (SM) (RUNX2 SM=1,59 0,20 *vs.* LE=1,48 0,14; OCN SM=2,57 0,28 *vs.* LE=2,78 0,27; BMP2 SM=1,55 0,19 *vs.* LE= 1,42 0,15); moreover, the addition of lenalidomide 10 uM to OM didn't increase osteogenic markers levels respect to OM alone (RUNX2 OM=2,86 0,25 *vs.* OM+LE=2,75 0,13; OCN OM=3,59 0,17 *vs.* OM+LE=4,04 0,19; BMP2 OM=2,84 0,28 *vs.* OM+LE= 2,64 0,26). In summary, our data show that Lenalidomide does not increase osteogenic markers' mRNA expression in BMSCs. Therefore its potential role in reducing MM bone disease is linked to its inhibitory effect on osteoclasts.

P188

REPETITIVE DNA METHYLATION IN MULTIPLE MYELOMA

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Multiple myeloma (MM) is a malignant proliferation of bone marrow plasma cells characterized by a wide spectrum of genetic and epigenetic changes. Global hypomethylation of repetitive genomic sequences such as long interspersed nuclear elements-1 (LINE-1) and Alu repetitive elements (approximately 500,000 and 1.4 million in the human genome) has been associated with chromosomal instability. Additionally, satellite alfa DNA (SAT-alfa DNA) hypomethylation has been reported to be associated to karyotypic instability in human cancer, possibly playing a role in centromere function. So far, the LINE-1/Alu and centromeric SAT-alfa DNA methylation patterns have not been investigated in the context of the different clinical and molecular MM subtypes. Global DNA methylation changes were investigated in a panel of 58 MMs at diagnosis, 7 plasma cell leukemias (PCL) and 6 healthy subjects as controls. DNA was extracted from purified plasma cells, treated with bisulfite and analyzed by bisulfite-PCR and Pyrosequencing. Methylation of LINE-1 and Alu elements was shown to correlate with total 5mC content and thus used to estimate global DNA methylation. MMs showed a decrease of Alu (20.1%) and LINE-1 (64.3%) methylation average levels compared with controls (26.2% and 84.5% respectively). Lower methylation average levels were also found in centromeric SAT-alfa DNA of MMs (69.9%) compared to controls (88.2%). The methylation average of PCLs was lower than MMs (16.8% versus 20.1% for Alu; 44% versus 64.3% for LINE-1; and 41.9% versus 69.9% for SAT-alfa DNA). Notably, a statistically significant association between SAT-alfa DNA and LINE-1 methylation (Pearson's correlations coefficient rho= 0.89; $p<0.001$) was found in MM. Finally, the comparison between methylation patterns and different molecular MM subgroups by means of the Wilcoxon Rank test, revealed that LINE-1, Alu and SAT-alfa DNA methylation was significantly lower in the nonhyperdiploid (P-value=0.034; 0.002 and 0.012 respectively) than hyperdiploid (HD) tumors. As regards the SAT-alfa DNA, a significant hypomethylation was also found in MMs with t(4;14) ($p=0.012$) whereas significantly higher methylation levels ($p=0.035$) were found in the TC2 group (HD tumors without primary IGH translocations and moderate levels of Cyclin D1). These findings suggest that our approach may contribute toward a more exhaustive stratification of the disease, potentially leading to a more effective patients management.

P189

CD200 EXPRESSION ON MYELOMA CELLS AND ANTITUMOR IMMUNITY

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Immune evasion in cancer plays a pivotal role in the failure of natural host antitumor immune response. CD200, initially described as the Ox-2 tumor antigen, is a cell surface ligand involved in regulating immune system. It is expressed on a variety of cell types, including myeloid cells, endothelium, ovarian cells, placental trophoblasts, and neurons where its interaction with CD200 receptors (CD200Rs) negatively regulates immune and inflammatory responses. Recent studies have identified CD200 as a downstream target of RAS/RAF/MEK/ERK activation in melanoma. In addition, CD200 expression was previously described on B cell lineage malignancies, and acute myeloid leukaemia. Moreaux et al. showed the correlation between CD200 mRNA expression and reduced event-free survival (14 months vs. 24 months) in patients with MM, independently from known adverse prognostic factors. Here we show that CD200 protein is over-expressed in 30 human myeloma samples. These cells expressed ERK but a low percentage (30%) expressed the phosphorilated form(p-ERK). In addition, we observed that CD200 MM cells have a reduced immunogenicity in Mixed Lymphocyte Reaction cultures in comparison with normal lymphocytes (CD25 expression <50%). We hypothesize that MM CD200 expression may suppress antitumor response and that anti-CD200 treatment might be therapeutically beneficial for treating CD200-expressing tumors.

P190

EVALUATION OF OSTEOCALCIN, CALCITONIN AND PARATHORMONE DURING THERAPY WITH BORTEZOMIB

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Several studies evaluated the behaviour of markers of bone metabolism in patients with Multiple Myeloma (MM) treated with Bortezomib. It has been observed that markers of osteoblastic activity (Alkaline Phosphatase, Osteocalcin) tend to raise after therapy. In some cases this trend is more evident in those patients with a better clinical response. In most cases these markers are dosed before therapy, after the first cycles and at the end of the treatment. In doing so it is difficult to distinguish between the direct effects of Bortezomib and those derived from eradication or control of the disease. In a previous work we measured the levels of Osteocalcin, Calcitonin and Parathormone (PTH) during therapy with Bortezomib and Dexamethasone, taking blood samples at every administration of the drugs. We noticed that already at day 4 there is a drop of Osteocalcin (72% *vs.* 100% of day 1), whereas levels of PTH tend to increase at day 8 (124%) and 11 (131%); Calcitonin on the other hand does not show any relevant variation. This behaviour has been observed without significant variations at every cycle. However we observed that in patients who had received only Bortezomib on day 1, there were not changes in Osteocalcin levels. In this study we have therefore verified these data increasing the number of patients who received only Bortezomib on day 1 (subsequently Bortezomib was administered with Desamethasone). 12 patients with relapsed/resistant MM were treated with Bortezomib and Desamethasone and received Bortezomib alone only on day 1 of first course. We dosed levels of Osteocalcin, Calcitonin

and PTH on day 1 and 4. Controls on day 4 showed: Osteocalcin-114% *vs.* 100% of day 1, Calcitonin-102.8%, PTH-90.8%. In a different group of 8 patients treated with Dexamethasone alone (40 mg/day, days 1-4) we registered on day 4 these levels: Osteocalcin- 37%, Calcitonin-68%, PTH- 336%. These results show that the inhibiting effect on osteocalcin is not linked to Bortezomib, but to combination with dexamethasone. Indeed, the latter has a similar activity when used as single agent. We confirm that Bortezomib does not seem to inhibit osteoblastic activity independently of the effects on plasmacells exerted during more courses of treatment of MM.

P191

ROLE OF THE PROTEIN INHIBITOR OF ACTIVATED STAT3 IN THE PATHOGENESIS OF MULTIPLE MYELOMA

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STAT3 is constitutively activated in a high proportion of primary myeloma cells and in human myeloma cell line. The production of Interleukin-6 (IL6), which has an essential role in multiple myeloma (MM), is strictly related to STAT3 expression. Recently, a new specific protein inhibitor of activated STAT3 (PIAS3) has been identified. PIAS3 blocks the DNA-binding activity of STAT3 and inhibits STAT3-mediated gene activation, suppress NF-kB transcription and modulates the cross–talk between STAT3 and other key cellular regulators. These evidences suggest a possible role of PIAS3 in the constitutive activation of STAT3 and/or in other signal transducers implicated in the pathogenesis of le myeloma (MM). Aim of this study was to evaluate PIAS3 expression in 3 types of myeloma plasma cells: in U266 cell line IL-6 dependent, in which STAT3 is constitutively activated, and in primary plasma cells enriched from MGUS (8) and MM patients (17). Primary myeloma cells were isolated by a positive $CD138^+$ selection with MACS system, whereas a combination of two techniques were adopted to isolate MGUS plasma cells: firstly a negative selection with RosetteSep to enrich a B cell population and then a positive selection with MACS system. Only samples with plasma cells content of at least 90% were used to perform Western blots analysis. PIAS3 was detected in U266 cell line, and in 13/17 samples of primary MM plasma cells. On the contrary, MGUS samples showed to be negative for the PIAS3 expression. This finding is not of univocal explanation. In fact, we found that in MGUS plasma cells there is a high deposition of proteins (probably light chains) which share the same region with PIAS3 that is 64 kDa. This interference might jeopardizes a clear detection of PIAS3. If the negativity of PIAS3 will be confirmed in further experiments, this would testify a crucial role of PIAS3 in the progression from MGUS to MM.

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DISULFIRAM INDUCES APOPTOSIS IN HUMAN MM CELLS

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Proteasome is a novel, interesting target in cancer drug therapy, and the proteasome inhibitor Bortezomib has documented effect in multiple myeloma (MM) patients. A recent work has described several compounds with proteasome-inhibiting activity, among which in the hit was the thiocarbamate alcohol-abuse deterrent disulfiram (DSF). DSF is an aldehyde dehydrogenase (ALDH) inhibitor. It was previously shown to block the P-glycoprotein extrusion pump, to inhibit the transcription factor nuclear factor-kappaB (NF-kB), to sensitize tumors to chemotherapy, to reduce angiogenesis, and to inhibit tumor growth in mice. Here we show that MM lell lines and primary cells from ten MM patients were significantly sensitive to DSF (DSF high dose induces about 45% of cell death after 48 hours of treatment). These cells are ALDH positive. When in combination with CuSO4, DSF has a cytotoxic effect *in vitro* at lower concentrations than disulfiram alone (DSF low dose plus CuSO4 induces about 75-80% of cell death after 48 hours of treatment). The apoptotic effect was comparable to that exert by therapy with bortezomib plus desametazone whereas DSF had only a weak effect on normal CD34 and peripheral blood mononuclear cells (<30% of cell death). In addition we show that NF-kB has a cytoplasmic inactive status in MM primary cells and DSF did not modify this localization. These data may suggest a novel strategy for treating MM by employing an old drug, with known side-effect, toward a new therapeutic use.

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CNS PROPHYLAXIS WITH INTRATHECAL LIPOSOMAL CYTARABINE IN PATIENTS WITH NON HODGKIN LYMPHOMA AND ACUTE LYMPHOBLASTIC LEUKEMIA

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Meningeal recurrence in aggressive NHL and ALL is an unfavourable event that occurs in up to 20% of the patients and often is followed by systemic relapse. It mostly depends on intensity and efficacy of frontline CNS prophylaxis. Liposomal cytarabine (lip araC) is a sustained release formulation of araC with a homogeneous distribution in the neuraxis and a prolonged half life, maintening cytotoxic concentrations in the CSF for more than 14 days. Our study aims to evaluate the safety and tolerability of lip araC in the CNS prophylaxis of NHL and ALL meningeal recurrences. 73 patients (16-77yo median 43,9) have been preventively treated with a total of 182 (range: 1-8) doses of lip araC 50 mg. Diagnosis consisted on 53 NHL: 30 high risk-CNS DLBCL (involvement of testes, paranasal sinuses, hard palate, orbit, paravertebral masses and bone marrow or IPI ≥2 with high level of LDH and ≥1 extranodal site involvement), 10 BL, 2 blastoid mantle cell, 7 lymphoblastic, 1PTCL, 1 gastric marginal zone lymphoma, 1 anaplastic, 1 follicular, and 13 ALL: 15 B-ALL, 4 T-ALL, 1 hybrid cells and 1 CML in lymphoid blast crisis. Five patients (1 DLBCL, 1 BL, 2 T-ALL, 1 B-ALL) received lip araC at their 1st systemic recurrence; of them, the DLBCL did not receive previous prophylaxis, instead of the T-ALLs had standard treatment with IT MTX; BL and B-ALL received not specified prophylactic therapy. All patients were treated according to the standard protocols in use for their disease. Five patients (2 DLBCL, 2 BL and 1 B-ALL) underwent autologous PBSCT. Four patients received IT or HD systemic MTX during treatment with lip araC. Seven patients received RT, as a part of prophylaxis program. All patients received lip araC 50 mg (and steroids) every 2, 3, 4 or 8 weeks, excepted 2 receiving 30 mg. A part of an episode of G2 headache and 21 cases of G1 headache, 10 episodes of G1 nausea/vomiting and 2 cases of localized or diffuse bone pain, no severe toxicity has been noted. So far, after a medium observation period of 17 months (1-34) only 3 patient (1 mantle cell lymphoma, 2BALL), showed CNS recurrence 10 months after diagnosis while in systemic relapse and died. None of the patients developed neurological symptoms or unexpected long term neurological side effects. Because of only few patients received CNS-directed concomitant therapy, lip araC appears effective towards CNS recurrence in the high risk NHL and ALL and well tolerated. More randomized studies are warranted.

P194

A VEGF-MEDIATED MOBILIZATION OF EPCS IS PRESENT IN POEMS SYNDROME AND CAN BE SUPPRESSED BY BEVACIZUMAB TREATMENT

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Several lines of evidence indicate that Vascular Endothelial Growth Factor (VEGF) can mobilize Endothelial Progenitor Cells (EPCs) from the bone marrow into the peripheral blood, both in animal models and in human subjects. Increased concentrations of VEGF are found in the plasma of patients with POEMS syndrome, and growing evidence suggests

a key role of VEGF in the pathogenesis of some clinical manifestations of this very rare condition. We had the opportunity to investigate the mobilization of circulating EPCs in two patients affected from POEMS syndrome who presented with high plasmatic concentrations of VEGF and were treated with Bevacizumab (Roche), an anti VEGF monoclonal antibody. VEGF plasma levels were assessed by ELISA and the frequency of circulating EPCs by *in vitro* culture of late outgrowing endothelial colonies, according to Ingram DA et al (Blood 2004; 104:2752). The endothelial origin of the colonies was confirmed by staining of the cells with anti-CD31, -CD105, -CD144, -CD146, -vWf, -VEGFR-2, -CD14 and -CD45 monoclonal antibodies and by *in vitro* capillary formation in the matrigel assay. Patients were treated with Bevacizumab, with infusions programmed according to VEGF levels and repeated until VEGF concentrations fell into reference values. At admission, VEGF plasma levels were 1756 and 3009 pg/mL, respectively (reference values 15-115 pg/ml) and the frequency of circulating EPCs 4.5 and 3/10^7 mononuclear cells (MNC), respectively (normal values in 12 normal age-matched healthy subjects: median 0.5/10^7 MNCs, range 0-1). Treatment with Bevacizumab induced a consensual reduction of plasma VEGF levels (98 and 42 pg/mL, respectively) and of circulating EPCs (0 and 0/10^7 MNC, respectively) in the two patients. Our data show that in POEMS patients high levels of plasma VEGF are associated with an increased frequency of circulating EPCs, assessed as late outgrowing colonies, and that treatment with an anti-VEGF monoclonal antibody induces a rapid, parallel decrease of both serum VEGF levels and of circulating EPCs. They also suggest that the neoangiogenetic processes described to be increased in POEMS patients and likely involved in the pathogenesis of some clinical manifestations can be due to the elevated frequency of circulating EPCs.

P195

FUNCTIONAL EFFECTS OF THE BCL-2/BCL-XL INHIBITOR ABT-737 ON PRIMARY CELLS FROM SMOLDERING AND SYMPTOMATIC MULTIPLE MYELOMA

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Smoldering multiple myeloma (SMM) is an asymptomatic plasma-cell disorder with a high risk of progression to a symptomatic multiple myeloma (MM). The time and mechanisms underlying progression are still unclear. The bcl-2 family proteins are frequently aberrantly expressed in MM and confer resistance to chemotherapy. The activity of inhibitors of the anti-apoptotic bcl-2 family members is currently under investigation in MM, while little is known on their therapeutic potential in SMM. Here, we investigated the functional effects of the bcl-2/bcl-xL inhibitor, ABT-737 (kindly provided by Abbott Laboratories), on primary $CD138^+$ malignant plasma cells from SMM samples, as well as from symptomatic MM at different stage of disease. MM cell lines exposed to increasing concentrations of ABT-737 (10-1000 nM) for up 72 h showed a dose- and time-dependent cell growth inhibition (IC50 200-400 nM at 72 hours) due to a significant increase in the proportion of apoptotic cells. In addition, cell cycle analysis demonstrated a significant G1-phase depletion in KMS18 cells exposed to 1000 nM ABT-737 for 72 hours (26.9%±1.4 *vs.* 54.6%±4.5, p=0.021). Western Blot analysis demonstrated that ABT-737 dramatically reduces bcl-2 protein levels, without affecting Bcl-xL. The effects of ABT-737 were then examined on primary cells from 9 smoldering MM. Cells from bone marrow aspirates were enriched for CD138 to a >80% purity and then cultured with ABT-737 (10-1000 nM) for 72 hours. A remarkable reduction in cell growth and increase in the percentage of apoptotic cells was observed in response to 100 nM ABT-737. In particular, a statistically significant pro-apoptotic activity of ABT-737 was demonstrated by the increment of the subG0/1 peak after 24 hours (25.0%±9.8 *vs.* 60.1%±17.0, p=0.00011, and 25.0%±9.8 *vs.* 78.1%±14.0, p=0.0003, in the presence of 100 and 1000 nM ABT-737, respectively). Similar results were obtained when $CD138^+$ cells from symptomatic MM (8 newly diagnosed and 8 relapsed/refractory) were exposed to the bcl-2/bcl-xL inhibitor, ABT-737. In conclusion, ABT-737 shows potent *in vitro* growth-inhibitory and pro-apoptotic activity in MM regardless of disease "stage" or "status". These data warrant further pre-clinical/clinical development of the bcl-2 family inhibitor in MM and suggest that such agents may also delay disease progression.

Thrombosis

P196

THE VON WILLEBRAND DISEASE TYPE 2A (IIH): A UNIQUE VARIANT OF VON WILLEBRAND FACTOR DUE TO COMBINED 2A(IIC)/2N DEFECTS

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Von Willebrand disease (VWD) type 2A/IIH is characterized by the loss of the high molecular weight multimers in plasma/platelets and of the "triplet structure" of von Willebrand factor (VWF) (Am J Hematol 1989; 32:287). We have recently found three missense mutations in the propositus: 604C>T (R202W, D1 domain), 2546G>A (C849Y, D' domain) and 4748G>A (R1583Q, A2 domain): R202W and R1583Q in the same allele being found in the propositus' daughter. Mutation 2546G>A is at last nucleotide of exon 19 and RT-PCR analysis showed an alternative splice site product which predicted the nonsense mutation C849X; mRNA sequences predicting both mutations C849Y and C849X were identified in patient's platelets. The three missense mutations were expressed alone, together and with the wild-type (WT). The amounts of recombinant (r)VWFs from mutants C849Y, C849Y/WT and hybrid R202W-R1583Q/C849Y rVWFs showed reduced levels in conditioned media and increased levels in cell lysates. A normal multimeric pattern was found in R1583Q rVWF, while mainly dimers and intermediate molecular weight multimers in R202W and C849Y respectively. Both hybrids R202W-R1583Q/WT and C849Y/WT rVWFs had an almost normal set of multimers, whereas hybrid R202W-R1583Q/C849Y rVWF showed markedly reduced multimerization with an intermediate pattern between R202W and C849Y multimers. To evaluate cleavage of VWF R1583Q by ADAMTS-13, two expression vectors, producing the WT or R1583Q VWFs A1-A2-A3 domains, were made (J Biol Chem 2007; 281: 30400). Digestion of both proteins with rADAMTS-13 showed a similar susceptibility to cleavage, not supporting an effective role of R1583Q mutation. Recently we found patient's plasma VWF to show a partially reduced VWF:FVIII binding, therefore C849Y rVWF was tested for its capacity to bind FVIII and resulted markedly reduced. Based on these findings *in vitro*, the type 2A/IIH VWD previously reported is actually due to combined type 2A/IIC (R202W) and type 2N (C849Y) defects since these mutations affect VWF multimerization, intracellular survival and VWF:FVIII binding.

P197

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

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Background. Clinical advantages of extensive screening for occult cancer in patients with idiopathic Deep Vein Thrombosis (DVT) is still debated since this approach improves early detection of cancer but not cancer-related mortality. Recently, we have demonstrated that patients with Residual Vein Thrombosis (RVT), 3 months after DVT, have a high risk for cancer in the subsequent 2 years (Siragusa S et al. Blood 2005;106(11):OC262). At the present it is unknown whether RVT assessment may be used to select patients, with idiopathic DVT, who require screening for occult cancer. Objective of the study. 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. All investigations had to be completed within four-weeks from the assessment of RVT. All patients were followed-up for at least 2 years. Incidence and cancer-related mortality was compared between the two groups by survival curves (Kaplan-Mayer) and related Breslow test for statistics. Results. Over a period of 6 years, 345 patients were included in the analysis: first cohort included 213 patients (Group A), second cohort 132 (Group B). Clinical characteristics between groups were homogenous. During the follow-up, 8.4% of patients developed overt cancer in group A; in group B, 8.3% of patients had diagnosed cancer at the moment of extensive screening while one new case (0.7%) occurred during the follow-up (Table 1). The sensitivity of this approach was 91.6% (95% confidence intervals 74.7-108.5). Cancer-related mortality was 6.5% in group A and 3.0% in group B (p<0.001). Conclusions. Our study demonstrates that RVT-based screening for occult cancer improves early detection as well as cancer-related mortality.

Table 1. Cancer events during surveillance.

Characteristic	*Group A (n= 213)*	*Group B (N= 132)*
Cancer, No (%) at screening time*	- -	**10 (8.3)
Cancer, No (%) during clinical surveillance**	18 (8.4)	1 (0.7)
Density incidence (cases x 1000 p/y)	41.9	40.3
Mean time cancer diagnosis (months±SD)	6.9 (1.3)	3.6 (0.7)
Cancer-related mortality, No (%)	14 (6.5)	4 (3.0)
Cancer-related mortality, mean time (months±SD)	19 (3.4)	18 (2.8)

**4+0.5 months. **At least 24 months*

P198

IMMATURE PLATELETS FRACTION (IPF): A SIMPLE TECHNIQUE TO PREDICT HAEMATOPOIETIC ENGRAFMENT AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Platelet regeneration represents an important and separate element in the engraftment process for allogeneic stem cell transplantation. The immature platelet fraction (IPF) as determined by flow cytometry is a rapid automated measure of the least mature component of the platelet population and is thought to correlate with thrombopoietic activity of the marrow. We investigated the ability and reliability of IPF to predict platelet recovery following hematopoietic progenitor cell (HPC) transplantation as engrafment picture. IPF was compared to standard parameters of hematopoietic recovery, including the immature reticulocyte fraction (IRF), an early predictor of recovery, absolute neutrophil counts (ANC) and platelets counts. We analyzed the kinetics of IPF in twenty-two patients undergone stem cell transplantation (SCT), nine autologous and thirteen allogeneic procedures. The source of allogeneic SCT was peripheral blood cells (PBSC) (eleven pts) and cord blood units (CBT) (two pts). Preconditioning therapy caused an immediate and rapid fall in tri-lineage hematopoiesis. Mean days to recovery for IPF was 2.4 days less than for platelet count, 3.8 days less than for ANC, and 0.8 days less than for IRF. IPF rose transiently above 5% after a mean duration of 11 days post-PBSCT, 19 days post-BMT and 24 days post-CBT. This was 2, 6 and 16 days earlier than platelet engraftment. IPF is comparable to IRF as early predictor of haematopoietic engrafment following stem cell transplantation. Then, IPF could potentially be useful as a predictor of engrafment to manage transfusion therapy.

P199

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 and *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 less than 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 follow-up (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 Table 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.

Table 1. Events between RVT negative and positive groups.

Outcomes	*RVT Neg. group (206)*	*RVT Pos. group (312)*	*p value*
Recurrences, n/total (%)*	2/206 (0.9)	63/312 (20.2)	<0.0005
Recurrences, n/100 person-year (%)*	2/184.7 (1.1)	63/191.3 (32.9)	<0.0005
Type of recurrent VTE			
DVT	1	43	
DVT + PE	0	6	
Isolated PE	0	3	
Controlateral	1	11	
Major bleeding, n/total (%)**	0/206	3/312 (0.9)	
Major bleeding, n/100 person-Yr (%)**	0/184.7	3/191.3 (1.5)	

**After OAT discontinuation, **During OAT.*

P200

MARKERS OF ANGIOGENESIS IN PATIENTS WITH VON WILLEBRAND DISEASE

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Objectives. Von Willebrand disease (VWD) is an inherited bleeding disorder characterized by a deficiency or abnormality of von Willebrand factor (VWF), the multimeric adhesive glycoprotein that plays a major role in platelet-adhesion and platelet-platelet interactions under high-shear stress conditions. VWF is synthesised in endothelium and magakaryocytes: it circulates in plasma and is present in subendothelium and platelet alpha-granules Emerging evidence underlines the connections between bone marrow derived stem cells and vascular wall formation: in particular, endothelial progenitor cells (EPCs) in angiogenesis and the presence of circulating endothelial cells (CECs) as marker of vessel formation/damage. VWF defects may therefore play a role at both these locations. To explore the role of VWF as marker of angiogenesis, we have evaluated the number of EPCs, CECs and various serum cytokines

in a cohort of 72 patients with different forms of VWD. *Patients and Methods.* Seventy-two VWD patients were diagnosed according to recommendations of the Scientific Standardization Committee on VWF of the ISTH. CECs ($CD146^+$, $CD31^+$, $CD45^-$) and EPCs ($CD34^+$, $CD133^+$, $CD45^-$) were evaluated by flow cytometry. Serum VEGF, TPO, sCD62E and EPO were determined by ELISA. RESULTS. VWD cohort was composed by the following VWD types: 1 (n=22), 2A (n=10), 2B (n=19), 2M (n=15), 3 (n=6). Additionally we examined 20 healthy controls. VWD population was characterized by high CECs (p=.0008) and low EPCs (p=0.001) numbers in comparison to controls. Moreover VWD patients had high VEGF (p=0.003), sCD62E (p=0.001) and EPO (p=0.0006) serum levels, while they showed normal TPO concentration. As far as VWD types, patients with VWD 1 were characterized by slightly high CECs (p=0.03), normal sCD62E and TPO, markedly low EPCs (p=.0005) and high VEGF (p=0.03); VWD type 2A and 2M grouped together showed high CECs (p=0.03) and low EPCs (p=0.007) as VWD type 1; high VEGF (p=0.006), TPO (p=.04) and markedly higher sCD62E (p<0.0001). Conversely, VWD type 2B by high CECs (p=0.0002), normal EPCs and elevated sCD62E (p=.002). *Conclusions.* Patients with VWD showed abnormal levels of serum EPCs, CECs, VEGF and EPO. VWD types characterized by reduced platelet-VWF interactions (Type 1, 2A and 2M) seem to involve bone marrow progenitors as shown by a marked decrease of EPCs. Conversely, VWD 2B with enhanced interaction with platelet glycoproteins, seem to involve primary the vascular cell compartments, as shown by increased CECs.

P201

ABNORMAL LARGE PLATELETS ARE ASSOCIATED NOT ONLY WITH VON WILLEBRAND DISEASE TYPE 2B BUT ALSO WITH SEVERE TYPE 3: THE ROLE OF VON WILLEBRAND FACTOR (VWF) DEFECTS IN THE IMPAIRED MEGAKARYOCYTOPOIESIS

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VWD type 2B results from mutations in exon 28 of the VWF gene. Gain of function of this adhesive protein results in an increased affinity for the platelet glycoprotein (GP) Ib-IX-V complex. Impaired megakaryocytopoiesis has been described in a family with the R1308P mutation (Blood 2006; 108:2587-95). *Aim of the study.* To examine the potential consequences of VWF abnormalities on platelet production we have studied the platelets of patients with different types of VWD. Patients, *Methods.* 13 VWD patients were enrolled in the study after signing their informed consent. Diagnoses of VWD were performed according to the criteria of ISTH-SSC-SC. 8 VWD 2B from 6 families with the following mutations: R1306W (n=1), R1308C (n=1), I1309V (n=1), V1316M (n=2), R1341Q (n=2) and P1266L (n=1, 2B/1NY). 5 additional VWD cases characterized by low/absent VWF in their platelets were also studied: VWD 2M (n=1, D1277-E78delInsL), VWD 3 without inhibitors against VWF (n=2, 276delT/257delA and 6182delT/6182delT) and VWD with alloantibodies against VWF (n=2, large deletions of the VWF gene). Electron microscopy (EM) and immunolocalization of VWF were performed. *Results.* EM showed the presence of an increased population of giant platelets (15 to 40% versus <10% for controls) in all VWD 2B. Additional abnormalities were observed in the patient with the 2B/1NY, alpha-granule morphology was different with a population of enlarged granules. Immunogold staining for all type 2B patients showed that VWF was present not only inside the granules but also in the Surface-Connected Canalicular System. For 3/8 patients with VWD 2B, cleaved caspase was present in the platelets indicating abnormal caspase activity at least for R1341Q and V1316M. In VWD 2M (n=1) and 3 (n=2) with a premature stop codon, no significant modification of platelet morphology was found. In contrast, a significant number of platelets with numerous vacuoles were found larger than controls in the two VWD 3 with large deletions and alloantibodies directed against VWF. Immunogold labelling for VWF was completely negative for these two patients. *Conclusions.* patients with VWD types 2B and 3 (undetectable VWF) show platelet production defects of varying severity, suggesting a major role of VWF in the fine regulation of megakaryocytopoiesis. Up-regulation or loss of the interaction between VWF and GPIb may lead to a variable proportion of giant platelets with or without thrombocytopenia.

P202

THE IMPACT OF DEEP VEIN THROMBOSISON ON OUTCOMES IN CRITICALLY ILL PATIENTS: A SYSTEMATIC REVIEW AND META-ANALYSIS

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Background. The clinical consequences of Deep Vein Thrombosis (DVT) have the potential to be serious yet are frequently unrecognized in the Intensive Care Unit (ICU). We hypothesized that both undetected and clinically evident VTE would affect the prognosis of critically ill patients Purpose: To systematically review whether a diagnosis of DVT in critically ill patients affects clinically important outcomes including length of stay, duration of mechanical ventilation and mortality. *Material and Methods.* Data sources used were the MEDLINE, EMBASE and PUBMED databases. Studies selected evaluated one or more of the following outcomes: duration of patient stay in hospital and in ICU, hospital and ICU mortality, and duration of mechanical ventilation. Two investigators independently extracted and reviewed data from each study; including study and patient characteristics and outcomes. Statistical heterogeneity was evaluated using the x2 statistic; Cohen's Kappa for inter-rater agreement was used to assess inter-rater reliability. Data was pooled using the Mantel-Haenszel method and a random effects model using Review Manager. *Results.* Five studies were included in the systematic review. Patients diagnosed with DVT compared to those without DVT had increased ICU and hospital stay (7.3 days (95% confidence interval [CI] 1.4 to 13.2; p=0.02) and 16.5 days (95% CI 1.51 to 30.59; p=0.03), respectively. Duration of mechanical ventilation was increased by 3.41 days (95 % CI –1.12 to 7.94; p=0.14). Patients diagnosed with DVT also had increased relative risk (RR) for ICU mortality of 9.19 (95% CI 1.07 to 78.65, p=0.04) and a trend towards increased hospital mortality (RR 14.32 [95% CI 0.59 to 347.96, p=0.10]). *Conclusions.* A diagnosis of DVT upon ICU admission appears to affect clinically important outcomes including length of ICU and hospital stay and ICU mortality. Further research involving larger prospective study designs are warranted.

Table 1.

Outcomes Study rate n)	*Duration of mechanical ventilation in days (DVT/NO DVT)*	*Hospitalization length In days (DVT/NO DVT)*	*ICU Stay In days (DVT/NO DVT)*	*Hospital mortality rate (DVT/NO DVT) n (%)*	*ICU mortality (DVT, n/NO DVT,*
Ibrahi 2002	m18.9±19.7/ 14.6±12.9 p=0.310	31.4±21.7/ 27.5±18.2 p=0.375	18.6±14.6/ 15.9±1.04 p=0.388	8.9 (34.6%)/ 26.8(32.1) p=0.815	n/a
Velmahos 1998	Not given. ^	49±32/	34±31/ 31±24 p≤0.05	n/a 19±18 p≤0.05	31%, 8.06/ 18%, 31.2
Major 2003	n/a	n/a	n/a	n/a	17%, 2/2%, 15 p=0.03
Patel 2005	n/a	26** (14,49)*/-	6** (3,15)*/-	70** (28.5%) [22.8-34.1])"/-	16.7%,41 [12.0-21.3]"/-
Cook 2005	9** (4,25)*/6 (3,13)* p=0.03	51** (24,73) */23 ** (12,47)* p≤0.001	17.5** (8.5, 30.5)*/9** (5,17)*	17 (53.1%)/85 (37.4%) p=0.04	-,8 **/-,62** p=0.78

PEPP: positive end-expiratory pressur; *IQR; **median;" [95%CI]); ^Necessity for ventilation measurated by PEEP; ≥10: DVT/no DVT: 11 (42%)/37 (21%).

P203

EXPANSION POTENTIAL OF PLATELET LYSATE ON MESENCHYMAL STEM CELLS ISOLATED FROM HUMAN UMBILICAL CORD, ADIPOSE TISSUE AND TRABECULAR BONE

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Background. Mesenchymal Stem Cells (MSCs) are multipotent, non-hematopoietic stem cells typically obtained from bone marrow but that can be isolated from many other tissues. They are characterised by their property to adhere to plastic, the presence of a pool of surface antigens (CD 105, CD 90, CD 44, CD 106, CD 73), their differentiation potential and immunosuppressive capacity. They are currently investigated for their clinical potential in cell therapy and tissue engineering. Most isolation and expansion protocols for clinical applications use Fetal Bovine Serum (FBS) as supplement, which possesses a potential risk for infections and immunological reactions. *Aims.* To find a suitable FBS substitute we investigated the effect of human Platelet Lysate (hPL) on expansion of human MSCs isolated from Adipose Tissue (AT), Trabecular Bone (TB), and Wharton's jelly of Umbilical Cord (UC) compared with FBS and human Platelet Poor Plasma (hPPP). *Methods.* MSCs isolated from the three sources (n=5 for all) were seeded at 1500 cells/cm^2 and cultured in DMEM supplemented with: 1) FBS (10%); 2) hPPP (10%); 3) hPL (10%). Cells were harvested, counted and analysed by flow cytometer every 7 days for 5 passages (P5). The population doubling (PD) was determined at each passage to calculate the cumulative PD (cPD); differentiation assays into adipogenic and osteogenic lineages were performed at the end of P2. *Result.* AT and TB derived MSCs showed a cPD significantly higher when cultured in hPL (20±2 for AT and 13±2 for TB at the end of P5) rather than in hPPP and FBS (8±3 and 6±3, respectively, for AT, and 6±2 in both conditions for TB [p<0,01]) (Figure 1). In contrast, UC-MSCs showed an explosive growth rate in all culture conditions (21±2 in hPL, 17±2 in hPPP and 15±2 in FBS). This proliferation can be attribute to their higher self-renew and proliferative potential than AT and TB cells. *Conclusions.* hPL represents an attractive candidate for MSCs expansion in clinical setting, since the large number of MSCs required for transplantation can be achieved in a shorter period of time. Further studies are necessary to determine immunosuppressive capacities of MSCs.

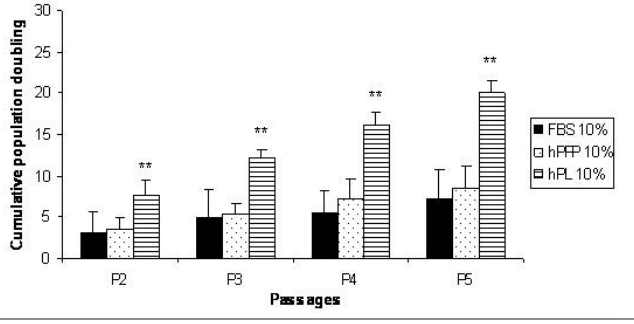

Figure 1. Expansion capacity of AT-MSCs cultured in FBS, hPPP or hPL. Data are expressed as mean±SD of five samples of each condition (, p<0,01).**

P204

RITUXIMAB IN THE TREATMENT OF A RELAPSING THROMBOTIC THROMBOCYTOPENIC PURPURA REFRACTORY TO PLASMA EXCHANGE AND STEROIDS

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Background. TTP is characterized by microangiopathic haemolytic anaemia, thrombocytopenia, neurological alterations, fever and renal failure. The pathophysiology is associated with ADAMTS 13 deficiency, frequently due to inhibitory autoantibodies. Treatment with continuous plasma infusion or therapeutic plasma exchange (TPE) has modified the prognosis, decreasing the mortality from 90% to less 20%. Nevertheless, one third of patients develops persistent or relapsing disease thus they need a complementary treatment including immunosuppressive agents. We report our experience about a patient affected with relapsing and refractory TTP treated by Rituximab, a anti-CD20 monoclonal antibody, after an unsuccessful therapy with TPE and steroids. *Case report.* A 24 years old woman, smoker and obese, suffered from TTP for 3 times (in 1999, 2001 and 2003) successfully managed with TPE; in april 2007, a new TTP relapse was observed. At diagnosis, the patient showed: fever, thrombocytopenia (PLT=28.000), anaemia (Hb=8,1) with peripheral schistocytes and a negative direct antiglobulin test, increased LDH (=4.957) and bilirubin, decreased aptoglobin, but without neurological symptoms and normal parameters of coagulation. The emergency treatment with methylprednisolone and TPE (carried out on consecutive day for 4 weeks and on alternative day for 2 weeks, with 1-1.3 plasma volume substitution using Fresh Frozen Plasma) was immediately started. After 7 days, ADAMTS 13 activity resulted <5% demonstrating the inhibitor's presence. After 14 TPE only a mild improvement of clinical conditions has been registered (PLT=44.000; LDH=2.286; Hb=8,4), thus Rituximab therapy was added (375 mg/m^2 weekly for 4 weeks). A complete remission (normal LDH and PLT with absence of symptoms for 15 days) has been achieved after 3 weeks of combined therapy, without no adverse effects. ADAMTS 13 activity was restored (>25%) after 3 months from the end of treatment and the remission is maintained until this date. *Conclusions.* A great variability in the strategies adopted for TTP's treatment is just nowadays present. Nevertheless the only therapy with proved efficacy is TPE, while complementary therapies may produce variable results; in fact numerous immunosuppressive drugs have been used in refractory or relapsing cases, with various success and side-effects. Our experience demonstrates that a combined therapy with TPE + Rituximab may be useful and safe in management of relapsing refractory TTP.

P205

MANAGING OF PLASMA EXCHANGE TREATMENT IN A PATIENT WITH THROMBOTIC THROMBOCYTOPAENIC PURPURA

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Background. Thrombotic thrombocytopenic purpura (TTP) or Moschowitz Syndrome is a disorder secondary to a deficiency of the ADAMTS13, a plasma zinc metalloprotease, that cleaves shear stress-activated von Willebrand factor, thereby preventing the occurrence of von Willebrand factor-platelet interaction in the circulation. The most successful therapy is the plasma exchange (PE) where the whole plasma volume of the patients is replaced by fresh frozen plasma (FFP) or cryosupernatant. Corticosteroid and/or immunosuppressive treatment are clinically utilized, too. *Aims.* In the present study we report a case of Moschcowitz's Syndrome with severe prognosis, that we have observed and attended from the 10th January, 2003. *Methods.* Plasmapheresis was the main treatment used. The PE was performed with cellular separator COMTEC (Fresenius), by exchange of 1 plasmatic volume /session and using as liquid of substitution FFP. *Results.* A 31-year-old woman, with diagnosed PTT, obtained a complete syndrome remission (CSR) after ten plasma exchange treatment, executed according to our protocols: 1treatment/die for seven days and subsequently to alternate days, for a total amount of 10 general sessions. In the 20th July 2006, the patient showed a relapse and the following values of laboratory analyses were identified: Hb: 9,5 g/dL; PLT: 4.9×10^6/mm^3; Aptoglobin: 5 mg/dL. Therefore, plasmapheresis therapy was immediately executed, in association with a corticosteroid/immunoglobulin treatment. At the end of the treatment a CSR was observed and the following values of laboratory were shown: Hb: 11,0 g/dL; PLT: 3,2×10^6/mm^3; Aptoglobin: 128 mg/dL. At the same time, a disappearance of the haemorrhagic and neurological symptoms was reported. At the present time, the patient is still alive, the disease's symptoms are absent and the parameters of laboratory are regular. *Conclusions.* In the recent years, therapeutic apheresis has dramatically improved the prognosis and outcome in patients with thrombotic thrombocytopenic purpura. Plasmapheresis is often resolutive and extremely effective for primary TTP. In this work, we describe a case of a TTP patient which condition have been resolved with plasmapheresis and cortison/immunoglobulin therapy. In particular, the PE treatment have been effective to induce the CSR in the acute phase of the illness. The analysis of this case suggests the need for an early diagnosis, even if this is not easy, in order to commence successful therapy.

Cell Therapy (I)

P206

CLINICAL SCALE SERUM-FREE EXPANSION OF HUMAN MESENCHYMAL STROMAL CELLS: VALIDATION OF A TWO-STEPS PROTOCOL UNDER GMP CONDITIONS

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Human multipotent mesenchymal stromal cells (hMSC) are considered good candidates for a growing spectrum of regenerative and immunomodulatory cellular therapies. hMSCs can be obtained relatively easily and expanded rapidly in colture. Nontheless, for clinical purposes, large numbers of cells are required so a rapid and efficent method of expansion should be established. In our AIFA approved Cell Factory, in compliance to Good Manufacturing Practice we have validated a two-steps protocol expansion starting from both bone marrow aspirates (35 to 60 mL) and washouts of bone marrow collection bags and filters. Briefly, total nucleated cells (from 485 to 1500×10^6) are collected and seeded without any further manipulation in 5% human platelet lysate (hPL) supplemented DMEM low glucose at 500.000 TNC/cm^2. After 48 hours nonadherent cells are removed and the adherent cells are expanded for 7-14 days with periodic feeding (twice weekly). The cells are then harvested (yield range from 7 to 20×10^6 hMSCs at p1) and seeded at low density (100-200 cells/cm^2) in 1 to 4 five-layered CellSTACK. The cells are allowed to expand for additional 10-20 days with periodic feeding and then harvested by addition of recombinant animal origin free Trypsin and frozen in 90% compatible plasma and 10% clinical grade DMSO in Hemofreeze bags. By seeding 0.6, 1.3 and 1.3×10^6 hMSCs (p1), we recovered respectively 96, 80 and 380×10^6 hMSCs. Furthermore, we also validated the possibility to freeze 2×10^6 hMSCs (p1) aliquots and subsequently thaw and expand. Indeed, in two experiments, yield of 96×10^6 and 250×10^6 were obtained by seeding 0.5×10^6 and 1.3×10^6 respectively in 10 days. Clinical scale expanded hMSCs identity and purity were assessed by testing viability and expression of CD14, CD45, CD73 and HLA-AB. Cytogenetic analysis was performed and no chromosomal alteration has been revealed. Bacterial, fungal, mycoplasma and endotoxin contamination were tested by validated tests and always found negative. This minimally manipulated two-steps culture procedure allowed us to produce sufficient numbers of hMSCs for therapeutic purposes using only clinical grade reagents and avoiding animal serum and to establish a frozen bank of early passages cells which can be subsequently recovered and further expanded. Furthermore it has been demostrated the normally discarded collection devices such as BM washouts represent a convenient source of large amount of hMSCs

P207

COMPARATIVE TRANSCRIPTIONAL PROFILE ANALYSIS OF HUMAN MESENCHYMAL STEM CELLS DERIVED FROM UMBELICAL CORD AND ADULT ADIPOSE TISSUE UNDER MULTIPLE CULTURE MEDIA

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Background. Human mesenchymal stem cells (hMSC) appear useful in cell-based therapies and clinical research for their plasticity and ability of self-renewal. In addition to bone marrow, different sources have been explored to make their use in clinics more feasible, possibly without the use of exogenous animal reagents. Clinical applications have been limited and the largest published data concern human bone marrow MSC (hBMSC) often expanded from fetal bovine serum (FBS). In this regard, it would be interesting to investigate the transcriptome signature that govern self-renewal, expansion and pluripotency of hMSC derived from different sources. *Aim*. To investigate the transcriptional profiles of human adipose tissue-derived MSC (hAMSC) and human umbelical cord-derived MSC (hUMSC) extracted from Wharton jelly under ex vivo expansion and differentiation in FBS-free media for clinical applications. *Methods.* hAMSC and hUMSC selected by their plastic-adherence and staminal immunophenotype were cultivated in the presence of FBS, human platelet poor plasma (hPPP) and human platelet lysate (hPL). Morphological adipogenic and osteogenic differentiations were obtained only for hAMSC_FBS and hAMSC_hPL conditions, respectively. hUMSC did not differentiate. For transcriptional analysis, primer pairs mapping in 3'-UTR of MMP2, GADPH, PPARG2, OCN and ALPL mRNAs were chosen and ABL as endogenous gene was used. cDNAs were analyzed by real time RT-PCR. *Results.* Table 1 summarizes most rilevant quantitative results. MMP2 exibited increased levels in both sources for all conditions. PPARG2 was highly upregulated in adipogenic differentiated hAMSC_FBS and over-expressed in hPPP and hPL media, too. PPARG2 in hUMSC was even greatly upregulated, despite morfological undifferentiation. Transcript levels for ALPL and OCN hAMSC_hPL agree with morphological osteogenic differentiation. ALPL was also upregulated for hAMSC_FBS, hAMSC_hPPP and hUMSC_FBS. *Conclusions.* PPARG2 regulates molecular signature of hBMSC, stimulating adipogenic toward osteogenic differentiation. Based on our results, similar consideration could be extended to hAMSC and hUMSC. PPARG2 overexpression role in hUMSC_FBS is under investigation. hAMSC and hUMSC MMP2 upregulation strongly candidate this gene as stemness-related in these sources, other than in hBMSC. The data presented underly the importance of molecular characterization of hUMSC and hAMSC as alternative MSC sources and of hPL as FBS-free medium for developing of new safe therapeutic strategies.

Table 1. Real time RT-PCR relative quantification (RQ) for hAMSC and hUMSC in FBS, hPL and hPPP supplemented media.

hAMSC (n = 4)										
Gene	*Reference*	*Sample*	*FBS*			*hPL*			*hPPP*	
			RQ	*RQ Min*	*RQ Max*	*RQ*	*RQ Min*	*RQ Max*	*RQ*	*RQ Min*	*RQ Max*
MMP2		BM	NC	14.3	7.9	25.8	8.7	6.5	10.0	6.7	5.5
8.9											
PPARG2	NC	AD	12.8	11.4	13.7	7.2	2.3	19.5	4.4	2.1	7.4
GADPH	NC	AD	2.7	2.2	3.4	0.8	0.6	1.0	1.5	0.7	2.2
OCN	NC	OS	1.0	0.9	1.1	2.6	1.2	4.2	0.8	0.7	0.9
ALPL	NC	OS	31.7	8.6	60.4	3.6	2.3	4.7	2.6	0.8	4.6
hUMSC (n = 4)										
Gene	*Reference*	*Sample*	*FBS*			*hPL*			*hPPP*	
			RQ	*RQ Min*	*RQ Max*	*RQ*	*RQ Min*	*RQ Max*	*RQ*	*RQ Min*	*RQ Max*
MMP2		BM	NC	16.0	10.0	21.7	20.8	13.4	31.2	15.4	9.7
21.0											
PPARG2	NC	AD	125.9	106.7	146.0	12.7	6.4	22.8	24.3	5.0	37.0
GADPH	NC	AD	0.8	0.5	1.5	0.3	0.2	0.4	0.4	0.4	0.5
OCN	NC	OS	1.9	1.5	2.8	1.2	0.6	1.8	1.4	0.8	1.6
ALPL	NC	OS	6.9	0.4	19.2	0.9	0.6	1.6	0.3	0.1	0.6

hAMSC, human adipose tissue-derived MSC; hUMSC, human MSC derived from Wharton jelly of the umbelical cord; FBS, fetal bovine serum, hPL, human platelet lysate, hPPP, human platelet poor plasma; MMP2, metalloproteinase-2; PPARG2, proliferator-activated receptor gamma-2 (adipogenic marker); GADPH, glyceraldehyde 3 phosphate dehydrogenase; OCN, osteocalcin (osteogenic marker); ALPL, alkaline phosphatase (osteogenic marker); BM, human bone marrow; NC, negative control (undifferentiated sample); AD, under adipogenic induction medium; OS, under osteogenic induction medium.

P208

IMMUNOSUPPRESSION MEDIATED BY BONE MARROW STROMAL CELLS IS DOWN-REGULATED BY 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE INHIBITORS

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Bone marrow stromal cells (BMSC) can exert an immunosuppressive effect by inhibiting proliferation and function of effector T lymphocytes. This inhibition is thought to be mediated by transforming growth factor-b, interleukin 10, prostaglandin E2 and indoleamine dioxigenase, but also BMSC to lymphocyte contact is needed. It has been proposed to infuse BMSC to improve bone marrow engraftment and to treat graft versus host disease. Thus, to use these cells, it is relevant to know how regulate their immunosuppressive effect. Herein, we have analyzed whether statins (i.e. atorvastatin, fluvastatin, mevastatin and simvastatin), reported inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-

Coenzyme A (HMG-CoA) reductase involved in cholesterol synthesis, may affect morphologic, phenotypic and functional features of BMSC. Upon exposure to any kind of statins tested, we observed striking morphological changes in cultured BMSC, with a decrease in adhesiveness and a complete detachment of cells from the substrate, that ultimately led to BMSC cell death. This was related to alterations in the assembly of actin microfilaments and microtubules, as indicated by confocal microscopy. Indeed, in statin-treated BMSC actin was localized at the periphery of the cell, close to the cell membrane while tubulin alpha and beta were not assembled into microtubule organizing centre. The S-phase of cell cycle was abrogated by statins and consequently BMSC division and proliferation was blocked. Statins could reduce the intensity of cell surface expression and distribution of ICAM1 and LFA3 adhesion molecules. As these molecules play a key role in the cross-talk between BMSC and lymphocytes, it is conceivable that BMSC-lymphocyte interactions are strongly affected by incubation of BMSC with statins. The statins-mediated effects were time and dose-dependent, as they occurred in the presence of 10 μM by 48h or after 72h with 1 microM concentration of statin The inhibition exerted by BMSC on T lymphocyte proliferation in response to phytohemoagglutinin A (PHA) or anti-CD3 monoclonal antibodies was strongly reduced when BMSC were pre-incubated with statins. Of note, mevalonic acid, the metabolic product of (HMG-CoA)-reductase could revert morphologic, phenotypic and functional effects of statins. Altogether, these findings indicate that alterations of cholesterol pathway can interfere with BMSC fate and thus statins may be useful in regulating BMSC-mediated immunosuppression.

P209

MESENCHYMAL CELLS RECRUIT AND REGULATE T REGULATORY CELLS

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Despite much investigation into T regulatory cells (Tregs), little is known about the mechanism controlling their recruitment and function. Since mesenchymal cells (MSCs) exert an immune regulatory function and suppress T cell proliferation, this in vitro study investigated their role in Treg recruitment and function. hMSCs and different T cell populations ($CD3^+$, $CD3^+/CD45RA^+$, $CD3^+/CD45RO^+$, $CD4^+/CD25^+$, $CD4^+/CD25^+/CD45RO^+$, $CD4^+/CD25^+/CD45RA^+$) from healthy donors were co-cultured for up to 15 days. Within $CD3^+$ fraction, percentages of $CD4^+$/CD25bright and $CD4^+$/CD25bright/$Foxp3^+$ cells were higher in the presence of hMSCs (42% *vs.* 34% for the $CD4^+$/CD25bright and 20.5 *vs.* 3.5 for the $CD4^+$/CD25bright/$Foxp3^+$). Within $CD3^+/CD45RA^+$ and $CD3^+/CD45RO^+$ fractions, the T reg starting fraction of the naïve population rose from 0.05%±0.01 CD4/CD25 positive cells to 0.2%±0.14 and the T reg starting fraction of the memory population rose from 0.3%±0.05 CD4/CD25 positive cells to 1.5%±0.9. When $CD4^+/CD25^+$, $CD4^+/CD25^+/CD45RA^+$, $CD4^+/CD25^+/CD45RO^+$ selected populations were used, cytofluorimetric analysis showed $CD4^+/CD25^+/FoxP3^+$ was up-regulated to 14%±4 and CD127 down-regulated to 4.4%±1.5 *vs.* respectively 0.2%±0.28 and 21.9%±3.5 in controls without MSCs. T regulatory inhibitory activity was lost within 15 days when sorted T regs were cultured without hMSC. On day $^+15$ proliferation using $CD4^+/CD25^+$ cells as suppressor cells was 1.7±0.8 *vs.* 45.2±12 in controls; proliferation using $CD4/CD25^+/CD45RA^+$ as suppressor cells was 5.4±3.1, *vs.* 21.3±11.2 in controls, and proliferation with $CD4^+/CD25^+/RO^+$ as suppressor cells was 2.3±2 *vs.* 33.5±8.7 in controls. We demonstrate MSC recruit Tregs from a fraction of $CD3^+$ and from immunoselected $CD3^+/CD45RA^+$ and $CD3^+/CD45RO^+$ fractions. After culture with MSCs both immunoselected fractions registered increases in the $CD4^+$/CD25bright/FoxP3 subset and CD127 expression was down-regulated. When purified Treg populations ($CD4/CD25^+$, $CD4/CD25^+/CD45RA^+$ and $CD4/CD25^+/CD45RO^+$) were used as fraction for hMSC co-cultures, they maintain FoxP3 expression and CD127 expression is down-regulated. Treg suppressive capacity was maintained in Treg populations that were layered on MSC for up to 15 days while control Tregs lost all suppressive activity after 5 days culture. In conclusion, our study demonstrates that MSCs recruit, regulate and maintain T regulatory phenotype and function over time.

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A FUNCTIONAL ROLE FOR SOLUBLE HLA-G ANTIGENS IN IMMUNE MODULATION MEDIATED BY MESENCHYMAL STROMAL CELLS FROM DIFFERENT SOURCES. EVIDENCE OF DISCORDANT REACTIVITY FOR VARIOUS IMMUNOLOGICAL MARKERS

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Biologic and clinical interest in mesenchymal stromal cells (MSC) has risen over the last years, mainly due to their immunosuppressive properties. Soluble factors produced by bone marrow (BM) MSCs have been suggested to play a fundamental role in mediating immune modulation. HLA-G antigens are MHC class Ib molecules characterized by a limited polymorphism and a splicing mechanism that regulates the production of membrane bound and soluble isoforms. IL-10 cytokine is one of the main up-modulators of soluble HLA-G antigen (sHLA-G) and increased IL-10 levels were reported as associated to MSC immune modulation. In our study we investigated , the possible role of sHLA-G molecules in the inhibition of the peripheral blood mononuclear cells (PBMCs) response to phytohemagglutinin (PHA) mediated by MSCs from different sources. We also investigated the basis of hMSC immunomodulant variability either in relation to the MSCs subset composition or to the different culture conditions, as well as culture passages. The results showed a significant correlation between the presence of increased levels of sHLA-G in the MSC/PBMC/PHA culture supernatants and lymphoproliferative inhibition. Neutralizing experiments performed with monoclonal antibodies directed against HLA-G and IL-10 molecules confirmed the inhibitory ability of sHLA-G antigens. Furthermore, exogenous IL-10 induced sHLA-G molecule secretion by MSCs alone in a polymorphic way, while a longitudinal analysis confirmed the loss of MSC inhibitory functions in relation to *in vitro* MSC aging. Our results further showed that hMSC from amniotic membrane, chorion and BM from patients with hematological malignancies (HM), showing a significant decrease in CD90 (Thy-1) surface molecule expression, elicited a lymphoproliferative allogeneic response in phytohemagglutinin (PHA)/PBMC cultures without any increase in sHLA-G and IL-10 levels. Overall the results obtained propose a functional role for sHLA-G molecules in inhibiting the PBMC response mediated by MSCs and strengthen the relationship between MSC activation, sHLA-G production and CD90 expression, suggesting the CD90 molecule as a novel predictive marker for hMSC inhibitory ability. These data may be of value in optimising MSC usage in transplantation and regenerative medicine settings.

P211

PHENOTYPICAL AND FUNCTIONAL ANALYSIS OF MESENCHYMAL STROMAL CELLS TRANSDUCED BY PMSCV-IRES–GFP RETROVIRAL VECTOR

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The capacity of mesenchymal stromal cells (MSC) to migrate from bone marrow or peripheral blood into damaged tissues renders these cells an attractive target for therapy for a broad range of diseases. The aim of our study was to develop and optimise a protocol to transduce MSC by using the pMSCV-IRES–GFP retroviral vector without affecting their phenotypical and functional features. MSCs were isolated from bone marrow of 4 healthy donors and infected, at the day 30 of colture, with different concentrations of viral supernatant, with or without subsequent spinoculation at 1600 rpm for 40'. After 72 hours of infection, MSCs were detached and the level of GFP was evaluated. The mean expression of GFP+MSCs transduced with high concentration of viral supernatant was 14% (range=8.3-19.8%, n=4) for non-spinoculated MSCs and 34% (range=11.9-56.2%, n=4) for spinoculated MSCs. The transduction efficiency of spinoculated MSCs slightly decreased when the cells were infected with low concentration of viral supernatant (mean of GFP+cells=18.9%, range=3.9%-27.9%, n=4). At the 4th passage, the percentage of GFP+MSCs was not different. The MSCs transduced with any method did not show any alteration in their phenotipical or proliferation properties. GFP+MSC expressed high levels of CD90,

CD73, CD105 (more than 80% for all markers and methods tested) and low level of CD45 and HLA-DR (less than 5% for both markers and methods tested). The quantification by RT-qPCR of chemokines, chemokine receptors and metalloprotease necessary to migrate into injured tissues, showed that the transduction process did not change the mRNA expression of these molecules. Further studies are ongoing to analyse the functional properties of GFP+sorted MSC. These results suggest that MSC were efficiently and stable transduced by pMSCV-IRES–GFP retroviral vector and that the transduction process did not alter the phenotype of MSC and the cytokine/chemokine network crucial for their immunoregulatory function.

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THE NUMBER OF FIBROBLAST CLONOGENIC PROGENITORS IS A PREDICTIVE VALUE FOR MESENCHYMAL STEM CELL *IN VITRO* AMPLIFICATION

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Adult mesenchymal stem cells (MSCs) can be easily expanded *in vitro* while maintaining their multipotency and, because of their low frequency in bone marrow (BM), the expansion is a prerequisite for clinical trials. It is known that fetal bovine serum (FBS) is essential to induce a significant *in vitro* MSC amplification, nevertheless, according to GMP requirements, the use of animal products should be avoided. In order to standardize a protocol eligible for clinical translation, we checked the activity of platelet lysate (PL), which contains several growth factors, and compared the effect of FBS and PL at different concentrations on MSC expansion. The cells obtained from 1-3 ml BM aspirate from healthy donors, or recovered from the filters wash out, were cultured with 5-10% PL or 10-20% FBS. The results were assessed in terms of total cell number recovered, immunophenotype, clonogenic potential and culture length. PL affected significantly the MSC expansion inducing a higher proliferation (Table 1). At the end of the P2, a significantly (p=0,023) higher MSC proliferation was detected in cultures supplemented with 5% PL compared to 10% FBS (DP 9.6±2 *vs.* 7.1±0.4), moreover the amplification time in PL medium was one week shorter. Despite the different cell morphology, no significant difference was detected between MSC amplified with PL or FBS in terms of immunophenotype and *in vitro* differentiation potential. Furthermore, PL induced more consistent results in term of DP time. In conclusion, PL medium enabled the generation of the cells required for *in vivo* human protocols in less than 4 weeks starting from a very small BM volume. In six experiments, to scale-up the expansion protocols, after the primoculture the cells were seeded in 632 cm^2 tissue culture flasks. The results previoulsly obtained in small flasks, were confirmed either in term of DP (8,3±2,7 *vs.* 9,1±2,8), time (34 *vs.* 35 days) or immunophenotype of the amplified cells. Interestingly, the number of colony forming units (CFU-F) detected in BM samples significantly correlated with the time to reach the confluence in primary culture: the primoculture length was 29,5; 17,5 and 13 days respectively for values of 0, <7 and >7 CFU-F out of 200.000 cells seeded. In conclusion, these results show that: the number of CFU-F in BM samples could be considered predictive for the amplification trend; platelet lysate is a powerful and safe substitute for FBS in MSC expansion for clinical setting.

Table 1.

	P2		
	Doubling population (DP)	*FI*	*Time (days)*
10% FBS	10% FBS	139,9±38,1	50
20% FBS	9,3±2,2	1384,5±1780,7	36
5% PL	9,6±2	1427,3±1362,5	43
10% PL	11,7±0,3	3288,7±744,7	37

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IDO-EXPRESSING DENDRITIC CELLS INHIBIT T-CELL ALLOREACTIVITY BY INDUCING A POPULATION OF REGULATORY T CELLS

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Indoleamine 2,3-dioxigenase (IDO) is the rate-limiting enzyme in the catabolism of tryptophan along the kynurenine pathway. IDO expression by different cell subsets, including dendritic cells (DCs), inhibit T-cell activation, proliferation and survival. IDO is a novel key player in graft-versus-host-disease (GVHD), where it reduces tissue infiltration and function of allogeneic T-cells. Recently, tolerogenic dendritic cells (DCs) have been shown to prevent GVHD by inducing a population of regulatory T cells (Tregs). In the present study, we characterized the expression and function of IDO by human dendritic cells after generation and maturation with different stimuli. DCs were generated from purified $CD14^+$ monocytes after culture with GM-CSF and IL-4 and then matured with CD40L, LPS alone, LPS plus IFN-gamma and a cytokine cocktail (IL-1beta, TNF-alpha, IL-6, PGE2). After culture, DCs were analyzed for IDO expression and function by real-time PCR, western immunoblot, kynurenine production, inhibition of allogenic proliferation and Tregs induction. Our results demonstrate that immature DCs have little if any expression of IDO. During maturation, the cytokine cocktail was the most effective in up-regulating IDO, both at mRNA and protein level, which was paralleled with higher kynurenine production and inhibition of allogeneic T cell proliferation. Moreover, DCs obtained after maturation with the cytokine cocktail had increased capacity to generate a population of $CD4^+CD25^+FOXP3^+$ Tregs from normal $CD3^+$ T cells. To test whether Tregs were capable to inhibit an *in vitro* model of GVHD, Tregs were added to cell-cultures consisting of T cells and allogeneic antigen-presenting cells. Tregs which had been generated by IDO-expressing DCs were capable to suppress allogeneic T-cell proliferation and this effect was abrogated by the addition of the IDO inhibitor 1-methyl tryptophan (1-MT). In conclusion, the cytokine cocktail is the best stimulus to induce IDO expression in DCs. IDO-expressing DCs induce a population of Tregs, which in turn are capable to suppress T-cell alloreactivity. These data have implications for the induction of T-cell tolerance during stem cell transplantation and may offer the rationale for novel approaches to GVHD prevention and management.

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DIFFERENT SOURCES AND SUPPLEMENTS MEDIA DO NOT INFLUENCE INHIBITORY EFFECT OF MESENCHYMAL STEM CELLS ON T CELL PROLIFERATION *IN VITRO*

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Background. Mesenchymal Stem Cells (MSCs) are multipotent cells characterized by specific surface antigens, the capacity to adhere plastic and to differentiate into different lineages. In addition, MSCs posses immunomodulatory activities that are being explored as a tool to control Graft versus Host Disease (GvHD), after allogeneic hematopoietic stem cells transplantation. Usually, this property is evaluated on Bone Marrow-derived MSCs in a medium with Fetal Bovine Serum (FBS). *Aims.* In this study we evaluated the inhibitory effect on T cell proliferation *in vitro* of MSCs isolated from different sources [Adipose Tissue (AT), Trabecular Bone (TB) and Wharton's jelly of Umbilical Cord (UC)] and cultured in 3 different supplemented media. Methods. AT, TB and UC-MSCs (n=5) were cultured in DMEM supplemented with human Platelet Poor Plasma (hPPP, 10%), human Platelet Lysate (hPL, 10%) or FBS (10%). At the end of passage 2 (P2) they were plated in 96 and 24 well plates in diminishing concentrations (20000, 10000, 1000, 100, 0 cells/well and 120000, 60000, 6000, 600, 0 cells/well, respectively). After 24 hours their proliferation was blocked with mitomycin C. Purified human T cells were added (10^5/well in 96 well plates and $6*10^5$/well in the upper chamber of a trans-well insert), at the beginning of culture and stimulated with phytohemagglutinin (PHA) for 7 days. T cells proliferation was measured with BrdU Proliferation Assay according to the manufacturer's protocol. Results. Suppression of lymphocyte proliferation was depended on the amount of MSCs in the 96-well plates in all media. The inhibitory effect was significant (p<0,01) when ratio was 1:10 (MSCs/T cells) for UC-MSCs and 1:5 for AT and TB (p<0,01, for both). In contrast, when MSCs and lymphocytes were separated by trans-well membranes, we did not measure a relevant decrease in T cells proliferation. Conclusions. These results suggests that, independently of MSCs sources and culture conditions, cell contact is fundamental for stem cells inhibitory effect. Furthermore, UC-MSCs present an higher capacity of inhibition *in vitro* than AT and TB; this could be attributed to their more undifferentiated status. The lack of inhibition in transwell experiments, in contrast with other published data, could be caused by the absence of MSCs activation and/or by the minor sensibility of our detection method. Other studies are necessary to verify which mechanisms are involved in the immunomodulation.

P215

EGF-PDGFBB-BFGF CAN SUBSTITUTE FBS AND PLATELET LYSATE IN EXPANSION OF ADIPOSE TISSUE –DERIVED MESENCHYMAL STEM CELLS

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Background. Adipose tissue (AT), like bone marrow, is derived from the mesenchyme and contains a heterogeneous cell population including Mesenchymal Stem Cells (MSCs). MSCs represent a promising tool for cell therapies because they are undifferentiated, able to self-renew, possess a mesodermal differentiation potential and immunosuppressive capacities. *Aim.* For clinical setting a large number of MSCs is required ($2*10^6$ cells/kg) so it is necessary an ex-vivo expansion. Usually clinical protocols report Fetal Bovine Serum (FBS) as medium supplement, but it is undesirable due to the risk of viral and prion diseases and immunological reactions. In this study we investigated the effect on AT-MSCs growth of FBS-free media containing: human Platelet Lysate (hPL); human Platelet Poor Plasma (hPPP) with and without Growth Factors (GFs) cocktail: Epidermal Growth Factor [EGF], basic-Fibroblast Growth Factor [bFGF] and Platelet-Derived Growth Factor-bb [PDGFbb]. *Methods.* MSCs were isolated from AT (about 200 cm^3, n=4) and cultured in DMEM supplemented with hPPP (10%) until the end of passage 0 (P0). Then cells were harvested and seeded at 1500 cells/cm^2 for 5 passages in media with: hPPP (3%) with and without GFs (all at 10 ng/mL), hPL (10%) and FBS (10%). At each passage we calculated the Population Doubling (PD) and the cumulative PD (cPD). Results. GFs cocktail supported AT-MSCs expansion better than hPPP at 3% (15±1 at the end of P5, and 5± 2 respectively) and FBS (9±1), further more it showed a cPD similar to hPL (19±1) (Figure 1). In addition surface markers expression did not differ markedly between the different culture conditions and remained stable. Conclusions. Starting from 200 cm^3 of AT and using GFs cocktail or hPL, we obtain already at the end of P2 a sufficient number of MSCs for clinical applications. hPL presents some limitations, in fact it contains aggregates of platelet membranes that are difficult to remove and adhere to AT-MSCs surface. In addition its use is limited by the amount of autologous Platelet Rich Plasma (aPRP) necessary to obtain hPL and to expand cells (about 300 mL of aPRP versus 90 mL of hPPP in our expansion conditions). The proposed cocktail represents an attractive candidate for MSCs growth. Further studies are in progress to determine its effects on differentiation and immunosuppressive potential.

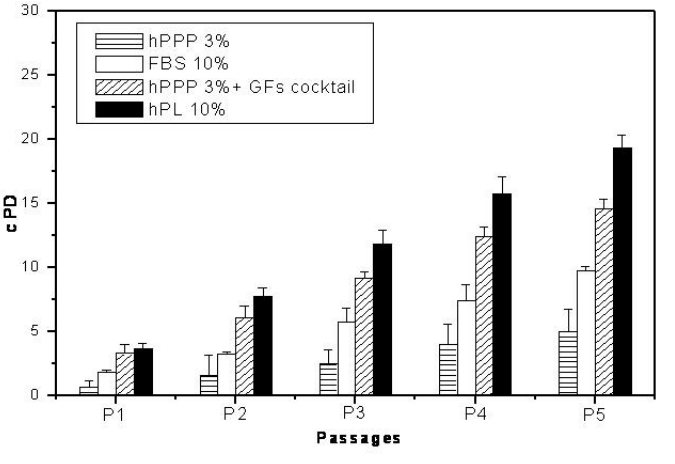

Figure 1. AT-MSCs expansion of 4 samples in the presence of hPPP (3%) with and without GFs cocktail (EGF-PDGFb-bFGF, all at 10 ng/mL), or FBS (10%), or hPL (10%).

Cell Therapy (II)

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ENDOTHELIAL CELL MIGRATION IMPAIRED IN DIABETIC TYPE 2 PATIENTS WITH SEVERE VASCULOPATHY

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Introduction. Remodelling of the endothelial cell basement membrane by MMP2 facilitates the endothelial cell migration. In setting of diabetic patients (pts), with or without vessel disease, we investigated MMP 2 and its correlated molecules such as follistatin-like 1 (FST) and connective tissue growth factor (CTGF). Moreover, we sought to evaluate other mediators and regulatory precursor involved in homing of endothelial cells: hypoxia-inducible factor 1, alpha subunit (HIF-1 alpha integrin beta 3 (ITGB3), integrin alpha V (ITAGV), EGF-like integrin (EDIL-3), chemokine (C-X-C motif) ligand 12 alias stromal cell-derived factor 1 (SDF 1-alfa). Patients and Methods. According with the cell therapy program in the Study Biology and Use of Endothelial Progenitor Cells in Peripheral Occluding Arteriopathy, approved by Ministry of Health, between February and April 2008, we enrolled 19 pts, type 2 diabetes less than 1year (group B) and 20 diabetic pts with older diagnosis, with peripheral arterial disease (group C). 26 healthy donors were as normal control group (group A). We used a TaqMan® Low Density Array based on comparative CTdd CT method on Applied Biosystems 7900HT to perform relative quantification of cDNA derived from PBMC samples. Results. Assumed that normal control values of all transcripts were=1, we observed that in B and C groups MMP2 was up-expressed (means 4.42, sd+12.3, 5.24 sd+6.7, respectively). FST and CTGF (means 11.9 sd+12.3, 4,5 sd+8.0, respectively). Among integrin expression levels, ITGB3 was normally controlled (means 1.30 sd+1,35 and 0.94 sd+0.95 in both B and C groups). ITAGV had weakly increased (means 4.4 sd+9.64 and 2.0 sd+2.3). EDIl-3 integrin showed opposite levels, increasing in group B (mean 6.4 sd+13.5), decreasing in group C (mean 0.8 sd+0.6). HIF-1 alpha has weakly up-regulated in group B (mean 4+2.4) and it has strongly increased in group C (13.0+17.7). These data agree with hypoxia environment documented in diabetic experimental animal models and in pts with diabetic retinopathy. More interesting was the lack of SDF 1-alfa expression in vascular compromised, group C (mean 0.52+1.1) versus group B (mean 3,7+7,7). In group B some subjects (9/19 pts) showed analogue SDF 1-alpha deficiency. Conclusion: In all diabetic pts, it appears that molecular permeability cross-talk may be up-established in response to increasing hypoxic promoter. Unfortunately, homing factor defect improves impaired angiogenesis in critical pts.

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ROLE OF AMNIOTIC FLUID MESENCHYMAL STEM CELLS IN CELL-BASED AND GENE THERAPY

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Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent stem cells, able to differentiate into multiple mesoderm-type cell lineages, including osteoblasts, chondrocytes, endothelial-cells, adipocytes, and also non-mesodermal lineages, like neuronal-like cells. MSCs were initially found in adult bone marrow (BM), but cells resembling BM-MSCs have been recovered from other foetal and adult tissues, including peripheral blood, liver, spleen, placenta, umbilical cord, amniotic membrane, adipose tissue and dental pulp. Due to MSCs diverse properties, clinical trials employing MSCs have been started for treating GvHD, heart disease, liver failure, Multiple Sclerosis, bone fractures and metabolic diseases. In a perspective of utilizing autologous cells in regenerative surgery and gene therapy for genetic disease, eventually during the prenatal age, finding alternative sources of MSCs is an imperative. MSCs cells can be retrieved from the amniocytes pool: so amniotic fluid, routinely collected by amniocentesis for prenatal cytogenetic diagnosis, could represent a valid source of autologous MSCs to employ in cell based and gene therapy. The aim of this study was to isolate and expand MSCs from amniotic fluid (AF), characterize their phenotype, assay their telomerase activity and test their transduction efficiency. AF specimens (n=11) were obtained by amniocentesis between the 15th and 18th gestational week. AF-MSCs were cultured under specific conditions for 8 weeks and analysed by flow cytometry and quantitative Real Time PCR, to assess the presence and expression levels of specific markers. Mesenchymal markers presented a peak of expression between the 3rd and 4th week of culture. After four weeks of *in vitro* culture we harvested 30-fold the initial number of cells. To assess the transducibility of AFMSC we used a SIN HIV-1 based lentiviral vector. We found a transduction efficiency reaching 60%. In conclusion, we demonstrate the presence of human multi-potent MSCs in second-trimester amniotic fluid. We suggest that AFMSCs may be good target for neonatal regenerative surgery and prenatal gene therapy, because of their ready expandability, ability to differentiate into multiple lineages and high transduction efficiency. Further studies are needed to evaluate the effect of transduction on MSCs self-renewal and differentiation. We are focusing on severe combined immunodeficiency due to adenosine deaminase deficiency as a model disease for gene and cell therapy.

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REQUIREMENTS FOR RETROVIRAL TARGETING OF A SUICIDE GENE TO ALLOREACTIVE SELF-RENEWING MEMORY LYMPHOCYTES FOR ADOPTIVE IMMUNOTHERAPY OF LEUKEMIA

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In a phase I/II clinical trial investigating the prophylactic infusion of suicide gene-modified donor T cells in the context of haploidentical hemopoietic cell transplantation (haplo-HCT) for the treatment of high-risk leukemia, we observed a rapid and effective immune reconstitution. After activation with anti-CD3 antibodies, genetic modification of donor T cells was accomplished with a retroviral vector encoding for the Herpes Simplex thymidine kinase (TK). *In vitro* before infusion and *in vivo*, TK^+ cells displayed an effector memory (EM) phenotype ($CD45RA^-CD62L^-$, $CD28^{\pm}CD27^+$, IL-$2^{\pm}$IFN-γ^+) and were responsive to opportunistic viruses, such as cytomegalovirus (CMV) and Epstein Barr virus (EBV). In the case of graft-versus-host disease (GvHD), the administration of the prodrug ganciclovir (GCV) was able to abrogate the disease. The graft-versus-leukemia (GvL) effect was substantial in patients transplanted in remission, but failed to cure patients in relapse. Gene targeting with retroviral vectors is limited to memory T cells. Central memory (CM) T cells ($CD45RA^-CD62L^+$, $CD28^+CD27^+$, IL-2^+IFN-$\gamma^{\pm}$) share many chracteristics with stem cells, namely the ability to self-renew and to differentiate into a progeny of effector. EM TK^+ cells have a reduced alloreactivity. Since the target antigens of the GvL effect are alloantigens and alloreactivity seems to be confined to memory T cells with stem cell-features, crucial to the success of the strategy is the suicide gene-modification of this subset. We found that addition of CD28 costimulation on cell-sized beads and the use of homeostatic cytokines, such as IL-7 and IL-15, generates central memory (CM) TK^+ cells. CM TK^+ cells are highly alloreactive, both *in vitro* and *in vivo* in a humanized animal model of GvHD based on the grafting of human skin onto NOD/scid mice. Interestingly, CM TK^+ cells express the IL7Ralpha, a marker

associated with the stem cell-features of memory T cells. Moreover, IL7Ralpha expression is maintained after stimulation with alloantigens. Stimulation of CM, but not EM TK^+ cells with autologous dendritic cells pulsed with restricted peptides from the minor histocompatibility alloantigen (mHag) HA-1 or H-Y efficiently induces mHag-specific effector T cells that lyse natural ligand expressing HLA-$A2^+$ targets. TK^+ mHag-specific effector T cells also lyse $mHag^+$HLA-$A2^+$ leukemic cells and, when infused in conditioned NOD/scid mice harboring human leukemia, significantly delay disease progression. Altogether, these data suggest that optimal T cell receptor triggering and homeostatic cytokines are required for retroviral targeting of a suicide gene to alloreactive memory stem T cells and warrant their use for a safe and powerful GvL effect.

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T CELL ORIGIN OF CYTOKINE INDUCED KILLER CELLS

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Cytokine induced killer (CIK) cells are $CD3^+CD56^+$ cells obtained by *in vitro* stimulation of circulating mononuclear cells with interferon gamma followed by anti-CD3 monoclonal antibody and expanded for 21-28 days in the presence of interleukin 2. CIK cells show considerable NK-like cytotoxic activity against tumor cells of several lineages, in particular hematologic neoplasms and potent anti-tumoral activity *in vivo* when injected in mice carrying either murine or human tumors with little GVHD. We have investigated the cellular origin of *in vitro* expanded CIK cells and characterized them phenotypically and functionally in comparison with "bona fide" T cells and NK cells also present at the end of CIK cultures, and with circulating $CD3^+CD56^+$ cells. Using immunophenotyping, cell sorting and CFSE staining, we have demonstrated that $CD3^+CD56^+$ CIK cells derive from proliferating $CD3^+CD56^-$ T cells and not from the few $CD3^+CD56^+$ present in the starting culture. Indeed $CD3^+CD56^+$ cells were shown to be non proliferating and could not therefore be expanded in culture. In contrast $CD3^+CD56^-$ cells, isolated either at the beginning of culture or at different times during expansion could proliferate and give rise to $CD3^+CD56^+$ cells. More precisely CIK precursor appear to be confined to the $CD3^+$CD56-$CD8^+CD4^-$ population. We have observed that CIK cells express the T cell receptor, mostly alfa-beta, in a polyclonal fashion, like normal T cells, as shown by Vbeta repertoire analysis. Except for NKG2D and CD56, CIK cells do not generally express NK cell markers, such as KIR receptors, NKG2A and NKp46. CIK cells only express NKp44 and NKp30, but to rather low levels compared to NK cells. Gene expression microarray analysis has also revealed that CIK cells share a larger number of genes with T cells present at the end of culture rather than with NK cells, confirming their T cell origin. *In vitro* generated CIK cells differ from circulating $CD3^+CD56^+$ cells in that they express high levels of NKG2D and CD56 and strong cytotoxicity for NK cell targets such as K562. Moreover circulating $CD3^+CD56^+$ cells generally display a skewed Vbeta repertoire with over representation of few clones. Interestingly PBMCs containing circulating $CD3^+CD56^+$ with strongly skewed Vbeta repertoire gave rise to polyclonal CIK cells after culture. These observations demonstrate that CIK cells arise from the polyclonal $CD3^+$CD56-$CD8^+$ T cells rather than from the $CD3^+CD56^+$ cells present in the starting culture.

P220

ANALISYS OF BONE MARROW HEMATOPOIETIC AND MESENCHYMAL PRECURSORS IN PATIENTS WITH AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic Lateral Sclerosis (ALS) is a lethal disease characterized by the relentless death of motor neurons. Its pathogenesis is probably multifactorial and includes gene defects, undetermined environmental factors, and the physiological cellular aging. The symptomatic disease is preceded by a preclinical period of unknown length and duration of the symptoms is quite dissimilar. These still unclear variables have made difficult the assessment of an effective treatment. Stem cell therapy, combined with pharmacological treatment, could be foreseen as the most promising strategy for restoring lost neuromotor function and/or preventing degeneration of motor neurons in ALS. Autologous stem cells may be easily obtained from patient bone marrow, but their normal functional behavior has still to be proven. In order to assess this issue, we analyzed the *in vitro* characteristics of bone marrow hematopoietic and stromal progenitors in 21 patients with ALS. To evaluate the mesenchymal compartment, fibroblast colony-forming cells (CFU-F) and mesenchymal stem cells (MSCs) obtained by plastic adherence were performed. Adipogenic and osteogenic differentiations of MSCs were also investigated. To test the functional properties of hematopoietic progenitors and their stromal cells supportive capacities, long term cultures (LTBMC) and colony forming unit (CFU) both from freshly isolated bone marrow and from the supernatant of the LTBMC were accomplished. The expression of collagen I, collagen IV, MMP-9 and TIMP1, that may play an important role in the maintenance of MSCs function, was evaluated by Real Time PCR. Since it has been demonstrated that Collagen IV is directly degraded by MMP-9 release, we also investigate the possible ALS pathological effect on the proteolytic activity of MMP-9 by gelatin zymography. No significant differences were observed between normal and ALS patients' hematopoietic compartment. Conversely, an initial reduced growth of MSCs in ALS patients was observed, even though the recovery of normal functions and growth after trypsinization suggested a delayed activation of mesenchymal precursors, rather than defective MSCs. The median number of colonies obtained in the CFU-F assay was lower in ALS patients compared to the normal controls but the difference in colonies number was not significant. A significant increased mRNA expression of collagen IV and MMP-9 was observed, while no differences were observed for TIMP1 and collagen I expression and for proteolic activity of MMP-9.

P221

FLOW CYTOMETRIC ANALYSIS FOR THE EVALUATION OF ENDOTHELIAL PROGENITOR CELL LEVELS IN HEALTHY DONORS COMPARED WITH DIABETIC PATIENTS

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Introduction. The evidence that Endothelial Progenitor Cells (EPC) circulating in the blood are the major factor promoting angiogenesis in the complex mechanism of tissue repair, induced many research teams to explore the pathophysiology of EPC. These cells are an extremely rare events in normal peripheral blood, representing about from 0.01% to 0.001% of peripheral mononuclear cells. Flow cytometric analysis is a widely used method for identification and enumeration of these cells, although consensus about immunophenotypes have not yet been established. *Aim.* According with the cell therapy program defined in the Study "Biology and Use of Endothelial Progenitor Cells in Peripheral Occluding Arteriopathy", approved by Ministry of Health, we have investigated the presence of EPC in healthy volunteers and patients with diabetes, the latter split into two groups: 1) with a diagnosis less than one year, 2) with diabetes and concomitant vascular complications. Patient and Methods. In the present work we used a BD FACSCanto, a high performance flow cytome-

ter that is able to identify up to six different fluorescence. Therefore, we were able to study in the same sample different cellular sub-populations. In particular, we identify mononuclear cells through FSC/SSC dot-plot, and we performed death cells exclusion by 7AAD staining. Then, in the living mononuclear cells we identify: EPCs ($CD34^+$/$VEGFR2^+$/$CD133^+$) and late-EPCs ($CD31^+$/$VEGFR2^+$/Ve-$Cadherin^+$). We compared the number of the above mentioned population in the peripheral blood of healthy donors (CTRL; n=26); recently-identified diabetic patients (Dia<1y; n=13); and long-time diabetic patients whit vascular complications (Dia>1y; n=17) (Table 1). Result. We found a slight decrement (not statistically significant) in the number of EPC in Dia>1y versus CTRL. Importantly, we found a dramatic drop in the number of late-EPC with respect to CTRL, both in Dia<1y (p<0.050) and in Dia>1y (p<0.001). If we consider the diabetic patients with a diagnosis less than one year, the significant decrease of EPC may indicate the presence of an early vascular damage. Considerations. It is known that in diabetic population the percentage of EPC in Bone Marrow are normal, but EPC circulating present a substantial reduction. Our data confirm the imbalance in the mechanism(s) of EPC-mediated angiogenesis and vasculogenesis.

Table 1. Immunophenotype of EPC evaluated between healthy donors and diabetic patients.

Immunophenotype	*A-Healthy donors (26)*	*B-Dia<1y (13)*	*C-Dia>1y (17)*	*p value*
EPC early (CD34+/CD133+/VEGFR2+) Mean±SD/mL	135±163	83±55	122±92	NS
PC late (CD31+/VEGFR2+/VeCadh+) Mean±SD/mL	3096±3217	1235±716	724±352	A *vs.* B p<0,05 A *vs.* C p<0,001

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PREDICTIVE VALUE OF GENE EXPRESSION ASSAY TO IDENTIFY DEGREE INFLAMMATORY ANGIOGENESIS IN DIABETIC PATIENTS WITH EARLY STATE DISEASE

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Rationale. Angiogenesis is involved in pathological conditions as tumor growth and inflammation. Development and spread of new capillaries is directed and regulated by a complex network of mechanism with control angiogenesis in positive or negative manner. An early inflammatory reaction has a defined pathology with increased angiogenesis including vascular dilation and disintegration of vascular wall component. In diabetic patients (pts) with advanced stages of chronic inflammatory disease, the tissue parenchyma is replaced by fibrosis and angiogenesis is suppressed. Aim. According with the cell therapy program in the Study "Biology and Use of Endothelial Progenitor Cells in Peripheral Occluding Arteriopathy", approved by Ministry of Health, we performed a comparative gene expression profile of helper T lymphocyte inflammatory cytokines and VEGF molecule family among two group of diabetic pts: with and without presence of severe vascular diseases, documented by scans. Our aim is to provide correlation of transcriptional profile with clinical outcomes and highlight on predictive index of probable vascular disease in first group of diabetic pts. *Patients and Methods.* Between February and April 2008, in the Study were enrolled 19 pts, type 2 diabetes less than 1 year (group B), and 20 diabetic pts with older diagnosis, affected by retinopathy and/or other angiopathies (group C). 26 healthy donors were as normal control group (group A). We used a TaqMan® Low Density Array based on comparative CTdd CT method on Applied Biosystems 7900HT to perform relative quantification of cDNA derived from PBMC samples. *Results.* All diabetic pts showed an increased expression of Th inflammatory transcriptome and of VEGF pattern than normal control. In group C the IL-17A, IL 21, IFN-gamma, IL-7 were higher expressed than group B, IL-8, IL-6 and TNF-alpha showed similar levels in group B and in group C (Table 1). By comparing the pattern of mRNA of VEGF family genes we found opposite expression. No difference was detected in diabetic pts relatively to VEGF-A (group B= mean 9,3 sd±15, group C=mean 8,2 sd±6,2), and VEGF-B (group B = mean 4,8 sd±4,3, group C= mean 5,2 sd±4,6). VEGF-C was reduced in pts with severe vessel damage (group B=mean 7,1 sd±13,9, group C=mean 0,9 sd±1,32, p<0.02). Conclusion. Focusing on inflammatory cytokines such as IL-17A, IL-21, IL-7 IFN gamma and VEGF-C genes could help to identify individual's predisposition to switch over pathological conditions.

Table 1. Gene expression profile: inflammatory angiogenesis. *CTdd CT* method (control value=1).

Common Name	*Group B Mean±SD*	*GroupC Mean±SD*
IL-17A	1,68±2,30	84,16±212
IL 21	33,00±4,7	175±337
IFN γ,	6,8±8,3	29±44,2
IL-7	2,7±4,7	11,2±33,9
IL-8	17,3±52	13,9±12,4
IL-6	20,7±56,1	11,4±10,9
TNF-α	9,8±16,9	6,9±7,4

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ALTERATE MOLECULAR ANGIOGENIC PATTERN IN EARLY DIAGNOSIS OF DIABETIC TYPE II PATIENTS

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Introduction. Angiogenesis and post-natal vascular remodelling are under control of some mediators: Angiopoietin-1 (ANGPT-1), Angiogenin (ANG). Transforming growth factor beta 1 (TGF Beta1), transforming growth factor A (TGF A), Fibroblast growth factor 1 and 2 (FGF-1, 2), Ephrin (Eph-B2), Granulin (GRN), Interleukin-8 (IL-8), are pro-angiogenic activators; Angiopoietin 2 (ANGPT 2), thrombospondin-1 and 2 (THBS-1,2), Tumor necrosis factor (ligand) superfamily, member 15 (TNFSF-15), and Vasohinibin (VASH-1) are inhibitors; ANGPT1 and ANGPT2 are ligands for TIE family receptors tyrosine kinases, TIE 1, TIE 2 (TEK). It is unclear how the imbalance of angiogenic homeostasis acts in hyperglycemic disease. To elucidate the molecular mechanisms underlying the angiogenic dysfunctions in diabetic patients (pts) with severe vascular diseases, we analysed the putative changes in the expression of transcripts, promoters and inhibitors, in two setting of pts. *Patients and Methods.* According with the cell therapy program in the Study "Biology and Use of Endothelial Progenitor Cells in Peripheral Occluding Arteriopathy", approved by Ministry of Health, between February and April 2008, we enrolled 19 pts, type 2 diabetes less than 1year (group B), and 20 diabetic pts with older diagnosis, with peripheral arterial disease (group C). 26 healthy donors were as normal control group (group A). We used a TaqMan® Low Density Array based on comparative CTdd CT method on Applied Biosystems 7900HT to perform relative quantification of cDNA derived from PBMC samples. *Results.* Quantitative molecular profile of the whole angiogenic complex showed that

hyperglycaemic state was associated with increased mRNA expression of all promoters and inhibitors, than normoglicemic state (Table 1). This suggests that angiogenic damage has already began in setting pts without clinical signs of vascular disease (Group B). Angiogenic receptors Tie 1,2 revealed increased levels, as other mediators (mean 6,27 sd+6,48, group B, mean 6,85 sd+11,98 group C). In group B only THBS-1 showed less alteration expression than group C (mean 1,95 sd+2,19 group B, 4,28 sd+8,62 group C). Among group B and C, THBS-2 was only molecular marker that stored normal expression. *Conclusion.* Our data induces to speculate that angiogenic homeostasis may critically change earliest onset diabetic disease. Monitoring molecular abnormality could identify pts that may prevent the long term progression of vessel-destroying disease.

Table 1. Gene expression profile: angiogenic network. *CTdd CT* method (control value=1).

Gene Bank ID	*Common name*	*Patients: Group B Means and standard deviations*	*Patients: Group C Means and standard deviations*
Hs00765775	ANGPT 1	4,65 sd±5,80	4,90 sd±8,20
Hs00265741	ANG	5,54 sd±7,55	3,80 sd±4,25
Hs99999918	TGF1 beta	4,38 sd±4,7	33,05 sd±2,22
Hs0060818	TGF A	7,09 sd±5,46	4,57 sd±4,77
Hs00963711	GRN	4,92 sd±4,70	4,00 sd±4,34
Hs00197064	FBLN5	5,12 sd±4,25	8,26 sd±10,68
Hs00265254	FGF-1	47 sd±21,00	27,00 sd±8,80
Hs00266645	FGF-2	1,72 sd±1,11	2,80 sd±1,30
Hs00362096	Eph-B2	4,49 sd±6,12	2,14 sd±2,39
Hs00174103	IL-8	17,31 sd±52,13	13,97 sd±12,47
Hs00169867	ANGPT 2	115,39 sd±128,745,38	112,66 sd±176,78
Hs00178500	TIE 1	3,33 sd±3,77	1,99 sd±1,64
Hs00176096	TEK (Tie 1,2)	6,27 sd±6,48	6,85 sd±11,98
Hs00170236	THBS-1	1,95 sd±2,19	4,28 sd±8,62
Hs01568063	THBS-2	0,80 sd±1,70	1,00 sd±1,50
Hs00353710	TNFSF-15	3,72 sd±2,29	3,52 sd±3,36
Hs00208609	VASH-1	6,11 sd±10,99	7,36 sd±10,09

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CIRCULATING ENDOTHELIAL CELLS AND VASCULAR DAMAGE: FLOW CYTOMETRIC ANALYSIS FOR THE EVALUATION OF LEVELS IN HEALTHY DONORS COMPARED WITH DIABETIC PATIENTS

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Introduction. Circulating endothelial cells (CECs) express considerable damage to the endothelial cell layer. They are present at very low frequency in healthy human blood (from $1x10^7$ to $1x10^5$ per leukocyte, 1–20 cells mL(-1) of venous blood), CECs are defined by morphological features and expression of endothelial markers. Flow cytometric analysis is a widely used method for identification and enumeration of these cells, but still there is not a general consensus regarding identification of CECs and their discrimination from endothelial progenitor cells. Aim. According with the cell therapy program defined in the Study "Biology and Use of Endothelial Progenitor Cells in Peripheral Occluding Arteriopathy", approved by Ministry of Health, we document and analyze numerical differences of CECs in the context of two populations of patients with diabetes: the first group with a diagnosis less than one year and a second group with vascular complications compared to a control group of healthy volunteers. *Patients and Methods.* We used a BD FACSCanto, a high performance flow cytometer that is able to identify up to six different fluorescence. In particular, we identify CECs through FSC/SSC dot-plot. Then, in the living mononuclear cells we identify: 1) live CECs ($CD45^-$, $CD146^+/CD31^+$), 2) activated live CECs ($CD45^-$, $CD146^+/CD31^+$, $CD106^+$). This immunophenotype was investigated in the peripheral blood of healthy donors (CTRL; n=21); diabetic patients with diagnosis than less one year (Dia<1y; n=19); and diabetic patients whit vascular disease (Dia>1y; n=19). Results. We have documented a statistically significant difference between the group of diabetics with vascular damage and other groups both for the number of alive CECs and alive and activated CECs. In particular, the number of activated CEC in patients newly diagnosed with diabetes presents a trend of significance compared to healthy volunteers to confirm a possible early vascular damage not yet evident with instrumental investigations (Table 1). *Consideration.* Our data could support the idea that the systemic vascular disease in the diabetic patients could find a possible early marker in the analysis of activated CECs, which might be used in monitoring the clinical development over time of diabetes.

Table 1. Immunophenotype of CECs compared between healthy donors and two group of diabetic patients.

Immunophenotype	*A- Healthy donors (21)*	*B- Dia<1y (19)*	*C- Dia>1y (19)*	*p value*
CEC ($CD45^-$, $CD146^+/CD31$) $p<0.001$ Mean±SD/mL	129±149	179±178	928±1000	C *vs.* A C *vs.* B $p<0.01$
CEC activated (CD45, $CD146^+/CD31^+$, $CD106^+$). $p<0.001$ Mean±SD/mL	1±2	30±33	92±98	C vs A $p<0.001$ C *vs.* B

Transplantation (I)

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PHASE I-II TRIAL OF ADOPTIVE IMMUNOTHERAPY WITH HAPLOIDENTICAL KIR LIGAND-MISMATCHED NATURAL KILLER CELLS IN HIGH RISK ACUTE MYELOBLASTIC LEUKEMIA PATIENTS

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The effector function of natural killer (NK) cells is regulated by activating and inhibitory receptors, termed killer immunoglobulin receptors (KIRs). In haploidentical T-cell depleted transplantation the donor/recipient KIR mismatch significantly impacts on NK-mediated tumor cell killing, particularly in acute myeloid leukemia (AML). Thirty-four high risk AML patients entered a phase I-II study of adoptive NK-cell based immunotherapy and were screened for the avalilability of one haploidentical KIR ligand mismatched donor. Eighteen of them resulted as having one suitable donor. NK cells were enriched from steady-state leukaphereses by using a double-step immunomagnetic separation system, consisting in depletion of $CD3^+$ T cells followed by positive selection of $CD56^+$ NK cells. $CD56^+$ NK cells were enriched from 8.28±3.7% to 85.9±3.2% (recovery 67.9±15 %, viability >92%). So far, 10 patients (1 partial remission, 4 progressions and 5 complete remissions) received NK cell infusion which was preceeded by immunosuppressive chemotherapy (fludarabine and cyclophosphamide) and followed by interleukin 2 injections. The median number of reinfused NK cells was 3.6×10^6/Kg and contaminating $CD3^+$ T cells were always less than 1×10^5/Kg. The procedure was well-tolerated and no significant toxicity, including GVHD, related to NK cell infusion was observed. The patient in partial remission obtained a complete remission, which lasted for 6 months; among the 4 patients with active/progressive disease 3 patients showed the persistence of disease and one patient died during the aplastic phase. Among the 5 patients in complete remission, 3 patients are stable after 12, 8 and 1 month of follow up, whereas 2 patients relapsed after 2 months. Biological studies demonstrated the presence of alloreactive NK cell clones in all the donors and in 3/4 patients. Donor NK cells were demonstrated in 2/5 patients with a peak at day 12 after infusion. Interestingly, a mixed chimerism was shown only in patients in CR. This clinical observation may be correlated with *in vitro* studies, indicating that AML cells are capable to induce NK cell apoptosis in a dose-dependent manner. In summary, a two-step enrichment of $CD56^+$ NK cells allows the collection of a suitable number of target cells to be used as adoptive immunotherapy in AML patients. Infusion of NK cells is feasible and safe and adoptively transferred NK cells can be detected in the peripheral blood after infusion.

P226

THYMIC OUTPUT AND T CELL RECONSTITUTION FOLLOWING NON-MYELOABLATIVE ALLOGRAFTING

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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 cells, that do not replicate with subsequent cell divisions. sjTRECs evaluation by quantitative PCR and the evaluation of naive and memory T cells by flow-cytometry are the most reliable tools to asses the thymic function. Overall, 55 patients, median age 51 (r 34-64) yrs, conditioned with low dose TBI (200 cGy), with/without fludarabine (90 mg/m^2 total), 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 yrs post-transplant. Briefly, the co-expression of the CD45 isoforms showed that the number of $CD4^+CD45RA^+CD45R0^+$ T cells reached median values of 65/uL by day 28 and 82/uL at 1 yr. They gradually increased to median values of 131/uL, 158/uL, 181/uL, and 286/uL, at 2, 3, 4, 5 yrs.Naive $CD4^+CD62L^+CD45RA^+$bright T cells and memory $CD4^+CD62L^+CD45R0^+$bright T cells showed a gradual increase up to 3 yrs post-transplant with median values of 882/uL and of 532/uL respectively. Median values of sjTREC copies/100 ng DNA in peripheral mononuclear cells (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/100 ng 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 in PMC and $CD4^+CD62L^+CD45RA^{+bright}$ T cells ($p<0.001$). Importantly, a 60 y/o patient thymectomised 10 yrs before transplant showed no thymic output and a very poor recovery of the TCR repertoire. Though a detectable thymic function persisted, the T cell reconstitution was rather slow especially during the first 2 yrs post-transplant. Statistical correlations of the thymic function with disease response and transplant complications, such as GVHD and infections, will be presented at the meeting.

P227

INTERLEUKIN-17-PRODUCING T CELLS: A NEW POTENTIAL PLAYER MEDIATING GRAFT VERSUS HOST PATHOLOGY IN PATIENTS UNDERGOING ALLOGENEIC STEM CELL TRANSPLANTION

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Graft-vs-host disease (GvHD) is a major obstacle to safe allogeneic haematopoietic stem cell transplantation (HSCT), leading to significant morbidity and mortality. The cellular and molecular mechanisms underlying GvHD pathophysiology are still poorly understood. Recently, a subset of interleukin (IL)-17-producing cells, named Th17, have been shown to play a central role in the induction of autoimmune-tissue injuries and inflammation. The aim of our study is to analyze the Th17 population in the Peripheral Blood of patients undergoing GvHD, after allogeneic HSCT. For this purpose, IL17A and IFNγ production and the phosphorylation of STAT-3/5 were evaluated in immunoselected-$CD4^+$ T cells, purified from PBMC of 5 GvHD patients. Moreover, the plasma level of IL17A was analyzed. As already described, an increased proportion of Th1 cells in GvHD patients in comparison with healthy donors (HD) (average fold increase, FI=2.6, range=2.0-5.1) was observed. Interestingly, an augmented population of Th17 (both IL17+/IFNγ and IL17+/$IFN\gamma^+$ producing cells) was detected in GvHD patients compared to HD (average FI=2.7; range=1.2-4.4). In particular, the percentage of IL17+/IFNgamma- Th17 cells increased from 0.4% (range=0.2-0.6%) to 1.2% (range=0.6-1.9%) and the percentage of IL17+/$IFN\gamma^+$ Th17 cells increased from 0.12% (range=0.12-0.13%) to 0.3% (range=0.1-0.6%). The enhanced Th17 population in GvHD patients was further confirmed by elispot assay (spot increase= 3.1 *vs.* HD). Of note, Th17 did not spontaneously produce IL17A, but only following polyclonal stimulation. In line with the already described molecular pathway involved in the Th17 differentiation, high phosphorylation of STAT-3 and 5 was observed. Finally, the detection of IL17A-plasma level showed that this cytokine was absent (<4 pg/mL) in both HD and GvHD patients. Further studies are ongoing to analyse the Th17 functions in tissues involved in GvHD pathogenesis. The better understanding of the role played by Th17 in the process of tissue-injury, could open the way to new highly targeted strategies for the management of GvHD.

P228

LARGE-SCALE SELECTION OF NATURAL T REGULATORY CELLS

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$CD4^+CD25^+$ regulatory T cells were isolated using large scale (CliniMACS Instruments, Miltenyi Biotec GmbH, Germany) separation systems using double negative selection (anti-CD8 and anti-CD19) followed by positive selection (anti-CD25). The initial leukapheresis products contained a median of 7.1×10^9 (range 4.2-9.2×10^9) nucleated cells. In the starting fraction the median number of $CD4^+/CD25^+$ was 238×10^6 (range 157-509) while the median number of $CD4^+/CD25^{+bright}$ was 102×10^6 (range 52-184). After magnetic cell separation a median of 186×10^6 (range 117-381×10^6) were recovered in the final cell fraction ($CD4^+/CD25^+$) with a mean purity of 93.6%±1. The efficiency of recovery was 81.52%±7.4. When $CD4^+/CD25^{+bright}$ are considered, a median of 55×10^6 (range 46-170×10^6) were recovered in the final cell fraction with a mean purity of 29%±7. The efficiency of recovery was 78%±20.9. Regulatory cells were identified not only by their high CD25 expression, but also by their high FoxP3 expression and low CD127 expression as compared with CD4/CD25- fraction. In particular the $CD4/CD25^+$ fraction contained a mean of FoxP3 cells equal to 51.9%±15.1; within $CD4/CD25^{+bright}$ fraction the mean of FoxP3 cells was 89.1%±3.4. The percentage of CD127 was also analysed. The $CD4/CD25^+$ fraction contained a mean of CD127 cells of 19%±11.5; within $CD4/CD25^{+bright}$ fraction the mean of CD127 cells was of 1.4%±1.4. Vβ spectratyping was performed on $CD4^+/CD25^+$ cells and $CD4^+/CD25^-$ cells. Complexity scores in T cells did not differ significantly (194±2 *vs.* 187±2), indicating that a broad T cell receptor repertoire had been maintained. The immune suppressive capacity of purified Treg populations was tested in an inhibition assay. $CD4/CD25^+$ cells inhibited $CD4^+/CD25^-$ cells (mean inhibition percentage: 52.1± 29.6% (ratio 1:1); 68±7(ratio 2:1)). In conclusion we demonstrated Tregs can be isolated under GMP conditions; the final product contained a mean of 93% $CD4/CD25^+$ cells, 29% of which were $CD4/CD25^{+bright}$; T-regs strongly expressed FoxP3 and were depleted of CD127 expression; they had a broad T cell repertoire; their suppressive activity is dose dependent.

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GENOME-WIDE GENE EXPRESSION PROFILING LIKE DIAGNOSTIC OR PREDICTIVE TOOL OF ACUTE GRAFT-VERSUS-HOST-DISEASE

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Background. Acute graft-versus-host disease (aGVHD) is the major complication after allogeneic haematopoietic stem cell transplantation. Modern approaches are needed to confirm clinical diagnosis of aGVHD and to predict the occurrence of this complication. Methods: We have analyzed expression changes of 48 genes associated with alloreactivity in 21 patients affected by haematological malignancies submitted to allogeneic stem cell transplantation from HLA-sibling (n=19) or MUD donors (n=2). We have used a TaqMan® Low Density Array based on comparative CTdd CT method on Applied Biosystems 7900HT to perform relative quantification of cDNA. In all patients serial samples of peripheral blood mononuclear cells (PBMC) were collected between 10-90 days after transplant and in each time when a clinical feature compatible with aGVHD was present. In 8 patients PBMC were collected also immediately before and after conditioning regimen used for the transplant. 10 patients did not experience aGVHD, 11 patients had II-III grade of aGVHD. Results: In patients with aGVHD, in samples collected during the complication a group of genes was strongly down-regulated. Molecules codified by these genes are implicated in cell survival functions (BCL2A1), transduction signals (IKB, NFkB), intercellular adhesion (ICAM-1), immune regulation (Fox-p3, CD52, CD83, CXCL1, CCL2, CCL5). When clinical manifestation was resulted, expression level of all these genes was increased. In patients without aGVHD, the gene expression profile was stable in the time. Between 8 patients analyzed before and after conditioning regimen, only one have accused grade III of intestinal aGVHD documented by histological analysis of bioptic specimen. In this patient, expression level of genes strongly down-regulated during aGVHD resulted strongly up-regulated in the sample collected after conditioning regimen. In the other 7 patients analyzed before and after regimen conditioning with did not have aGVHD, no changes were documented in expression level of the same genes. Conclusion: Our results demonstrate that our method can be an useful diagnostic tool to confirm aGVHD. It is necessary to increase the number of patients included in the study to sustain the importance of gene expression profile in samples collected immediately after regimen conditioning to predict aGVHD.

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CTLA-4 +49 A>G POLYMORPHISM OF RECIPIENTS OF HLA-MATCHED SIBLING ALLOGENEIC STEM CELL TRANSPLANTATION IS ASSOCIATED WITH SURVIVAL AND RELAPSE INCIDENCE

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Cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene behaves as negative regulator of T-cell activation. CTLA-4 gene polymorphisms have been found associated with several autoimmune disorders and, recently, with the outcome of allogeneic hematopoietic stem-cells transplantation (allo-HSCT). Perez-Garcia et al. (Blood, 2007) investigated CTLA-4 polymorphisms in HLA-matched sibling donor HSCT: the presence of at least one G-allele of CT60A>G polymorphism in the donors was associated with reduced overall survival (OS), higher relapse and lower risk of acute graft-versus-host disease (GvHD). Vannucchi et al. (Bone marrow transplant, 2007) confirmed the association with GvHD but not with OS and relapse in unrelated donor HSCT, whereas Azarian et al. (Blood, 2007) found only the association of chronic GvHD with donor +49A>G GG genotype in HLA-matched sibling HSCT. We have genotyped by tetra-ARMS-PCR 3 polymorphisms of CTLA-4 gene (-318C>T, +49A>G, CT60A>G) in 133 donor/recipient pairs from a single center who underwent HLA-matched sibling donor HSCT for hematological malignancies. Genotype frequencies of all 3 polymorphisms fit Hardy-Weinberg equilibrium in both recipients and donors. Transplant outcome was not associated with -318C>T and CT60A>G polymorphisms in either recipient or donor. In contrast with previous reports, a longer OS was associated with +49A>G GG homozygote donor (5-year OS: AA, 51%; AG, 46%; GG, 87%. p=0.04). Furthermore, we found that the number of recipient +49A>G G-alleles was significantly associated with longer OS (5-year OS: AA, 46%; AG, 55%; GG, 90%. P for trend=0.027), longer disease free survival (5-year DFS: AA, 59%; AG, 68%; GG, 80%. P for trend=0.036) and reduced relapse rate (AA, 43%; AG, 28%; GG, 20%. P for trend=0.042). Donor and recipient +49A>G polymorphism was not associated with either acute or chronic GvHD. Multivariate Cox's regression analysis confirmed the independent prognostic significance only of recipient +49A>G genotypes for both OS (P for trend=0.007) and DFS (P for trend=0.053). Our data show that recipient CTLA-4 +49A>G polymorphism, which has not been investigated so far, appears to be relevant for the clinical outcome of HSCT: this suggests that CTLA-4 expression on leukemic cells and on recipient micro-environment cells might play a role in post-transplant control of disease. In contrast with previous report, we found no effect of donor +49A>G and CT60A>G. The different clinical features of patients included in the different studies may partially explain these conflicting findings. Overall these discrepancies suggest strong caution in evaluating the possible impact of CTLA-4 polymorphisms on the clinical outcome of allo-HSCT: however, they warrant further investigations in proper designed prospective clinical trials.

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OPTIMISED BONE MARROW HARVEST RESULT IN BETTER OVERALL SURVIVAL AND IN EQUIVALENT ENGRAFTMENT TIMES IN RESPECT TO TRANSPLANTS DONE USING PBSC SOURCE

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Number of infused HSC is though to have an important effect after BMT on patient's survival, patients receiving a number of $CD34^+$ cells below median having a lower survival (H. Bittencourt et al Blood 2002). In order to increase the median number of CD34 cells infused and improve patient survival we have optimized our bone marrow harvest techniques. Aim of our study was to compare characteristics of inoculum, engraftment times and clinical outcome of two groups of patients: 1) those who received BM graft (harvested using "optimized" Bone Marrow Harvest) and 2) patients who received PBSC graft. A group of 60 consecutive allogeneic patients was studied. Patients received HLA identical transplant from sibling for various malignant diseases, BM (n:34) or PBSC assignment (n:26) to either source group was decided based on disease status and on donor sex. Optimization of BM Harvests was obtained using large and multi-bore needle (11 g), small volume of aspiration (3-5 mL), performing 5-10 skin punctures for each side and allowing only to experienced physician to perform the procedure. PBSC harvest was done after mobilization with G-CSF at 10 mcg/Kg day with a target infusion dose of 5×10^6/Kg $CD34^+$. Median donor weight was 74,5 Kg in BM group and it was 71,2 Kg in PBSC group (t test: p=0,6). GVHD prophylaxis was done in both groups with CSA plus MTX and median number of MTX doses was not different in BM and PBSC groups (3,41 versus 3,48). Median number of $CD34^+$ cells harvested from BM group was 6.0×10^6/Kg recipient weight (range 2.4-17.8) and it was 5.6×10^6/Kg recipient weight in PBSC group (range 3,4-10.2) p= 0.6. On the contrary Median number of TNC/Kg recipient weight in BM group was significantly lower in respect to what found in PBSC: 4.1×10^8/ (range 2.9-7.3) versus 6.1×10^8/ Kg (range 3-12.9) p=0.0009. Engraftment time to 0.5 $\times10^9$/L N. were comparable in the two groups 18.3 days in BM and 18.0 days in PBSC group (log rank: p=0.4). A count of PLT> 50.000 was reached faster, in PBSC group (median 15 days) in respect to BM (median 18 days) (Log rank: p=0.08), TRM at 1 year was 4.7% in BM group (KM estimated) while 27.3 % in PBSC. In patients transplanted in early phase of their disease, OS was 80% in BM group and 70% in PBSC group (log-rank p=0.4) while in advanced phase patients OS was significantly better after BM transplantation with respect of PBSC (65% versus 20%: log rank: p=0.01). In conclusion our optimized BM harvest can results in a CD34 content and in a myeloid engraftment time equivalent to what is possible to be obtained with PBSC. The improvement in OS observed in patients transplanted using BM harvest may derive from the optimization of the harvest technique and from the high CD34 content that we have been able to infuse.

P232

IMMUNE RECONSTITUTION AFTER ALLOGENEIC TRANSPLANTATION OF PERIPHERAL BLOOD STEM CELLS IN PATIENTS WITH MULTIPLE MYELOMA

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Background. Allogeneic transplantation of G-CSF-mobilized peripheral blood stem cells (PBSC) results in rapid and complete engraftment in a large proportion of patients and in relatively fast immune recovery. We have analyzed by flow cytometry the immune reconstitution in 19 patients (pts) affected by multiple myeloma undergone to allogeneic PBSC transplant from HLA-identical related donors. Methods: 19 pts (9 males and 10 females), median age 52 years (range 40-62), were undergone to allogeneic PSBC transplant after nonmyeloablative conditioning regimen with fludarabine 30 mg/m^2 and cyclophosphamide 300 mg/m^2 for three days. In each patient a comparable number of mononuclear cells, $CD3^+$ T lymphocytes and $CD34^+$ progenitor cells was infused. To evaluate the kinetics of the immune reconstitution, the overall number of total lymphocytes, T, B and NK cells of each patient were assessed before and 1, 2, 3, 6, 12, 18, 24, 30, 36 months after allogeneic PBSC transplant. However, of 19 pts included in the study, the analysis of lymphocyte recovery was performed at all established time in 17 pts. In one patient the analysis was performed until 1 month and in another until 6 months for transplant related mortality. Results: Overall T cell reconstitution was in all the pts at 3 months, since at that time the $CD3^+$ T cell median number was 880 cells/microl (r. 589-1357). However, in all pts high numbers of $CD3^+$ T cells were achieved at 12 months after transplant (median 1326 cells/microl, r. 850-2309). The $CD4^+$ T cell median number was 281 cells/microl (r. 185-433) at 6 months, 391 cells/microl (r. 303-505) at 12 months, 603 cells/microl (r. 433-736) at 18 months. The $CD8^+$ T cell median number was increased from the transplant to 18 months in which it was 1489 cells/microl (r. 760-1976). The decrease of $CD8^+$ T cells with the normalization of $CD4^+/CD8^+$ ratio was observed at 30 months when $CD4^+$ T cells were 650 cells/microl (r. 370-989) and $CD8^+$ T cells were 690 cells/microl (r. 445-1743). B cells recovery was observed at 18 months with a median number of 194 cells/microl (r. 40-404). The faster reconstitution was documented for NK cells with a median number of 314 cells/microl (r. 61-647) at 2 months. Conclusion: the complete immune reconstitution in our pts was achieved at 30 months after transplant. Our objective is to evaluate if this slow immune recovery is associated with a high incidence of infectious diseases and a low incidence of chronic GVHD.

P233

GENE EXPRESSION PROFILE ANALYSIS AS TOOL CONTROLLING THE HAEMATOLOGICAL DISEASE BEHAVIOUR OF PATIENTS AFTER NON MYELOABLATIVE ALLOGENEIC TRANSPLANT. PRELIMINARY DATA OF A SINGLE INSTITUTION

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Introduction. The goal of allogeneic transplant in haematological patients (pts) is the induction of persistent graft anti-tumor activity (GvT), with low incidence of GvHD. Based on the hypothesis that a component of GvT effect can be realized from type II immunity, with concomitant GvHD regulation, we performed a gene expression profile (GEP) analysis to control the Th2 immunotrascripts in a setting of patients with haematological diseases, underwent to allogeneic transplant with non myeloablative conditioning regimen and peripheral blood stem cells. *Patients.* We assessed 10 (pts) with poor haematological malignant diseases and allo-transplanted in according with Stanford protocol1. *Material and Methods.* We used a TaqMan® Low Density Array based on comparative CTdd CT method on Applied Biosystems 7900HT to perform relative quantification of cDNA. We tested PBMC samples collected at baseline, engraftment and sequentially after transplant. GEP study before and after start of steroid therapy was investigated in case of aGvHD. Results. The median follow-up was 255 days (r.175-400). Two pts, transplanted with active disease, died for Richter syndrome (RS) and aGvHD with brain bleeding, respectively. The others were alive

and in complete remission. Notably, at engraftment time the mRNA expression of type I and II immunity was down-regulated *vs.* normal control. Subsequently, variation of expression levels were detectable. We observed a prolonged and stable up-regulation of IL 4, IL 10, IL 6, IL 18 and of immunoregulatory mediators such as FoxP3 and CD83 in 8 pts with CR. The patient died with RS showed a dramatic decreasing of Th2 response. In 3 out 8 pts developed moderate aGvHD, the total immune response was disregulated with respect to their baseline value: IL-10, IL-18, Il-6, IL-4, FoxP 3,TNF-alfa, NFKB2, IFN-gamma, IL-12A, IL 12B, IL 1B. As a result of steroid therapy, expression level of all these genes was subsequently increased with restore of both Th2, Th1 effectors and Treg, too. In fatal aGvHD case we found a critical imbalance of all these cytokines with major Th1 influence (IFN-gamma) (Table 1). *Conclusion.* These preliminary data documenting that GEP study helps to know Th2 effectors regulating the immunological outcome of allogeneic transplant. Our analysis method could highlights many potential molecular targets for accurate monitoring of GvT. Of course, the inclusion of other cases is needed to support these results.

Reference

1. NEJM 2005; 353: 1321-31.

Table 1. The 12 genes with most important variability during immune alloresponse.

Common name sive	*Data analysis: Engraftment*	*Data analysis: n°3 pts aGvhd*	*Data analysis: n°3 pts Post-aGvhd*	*Data analysis: fatal aGvHD*	*Data analysis: Follow-up*	*Data analysis: fatal progres-*
	(mean)	*(mean)*	*follow-up (mean)*		*GvT*	*disease*
CD83	0,15989724	0,265 SD 0,49	1,49E+07 SD 1,48 E+07	0,003	4,18E+06 SD 1,21E+07	24,2
FOXP3-	0,44745934	1,1 SD 1,6	1,88E+06, SD 4E+06	0,4	9,87 E+06 SD 1,68 E+07	347
IL10	0,74507105	9 SD 11	4,19E+11 SD 1,66E13	1,75E+07	1,5E+07 SD 1,87E+07	0,65
IL18	0,9455	0,6 SD 1,7	1,84E+07 SD 2,81E+07	0,15	3,31E+08 SD 8,5E+08	1
IL1B	0,011780687	4,85 SD 6,25	1,33E+07 SD 1,24E+07	0,005	924E+06 SD 1,88E+07	0,17
IL6	0,00219748	2,25 SD 3,3	8,36E+06 SD 1,76E+07	3,09E+07	2,18E+07 SD 2,2E+07	15,6
IL12A	0,035014536	7,35 SD 9,17	9,8E+10 SD 1,51E+07	1,18E+07	7,61E+06 SD 1,44E+07	4,86
IL12B	0,743566	1,68 SD 3,32	9,97E+06 SD 1,26E+07	9,51E+06	3,19E+13 SD 1,04E+013	89,8
IL4	0,299192	1,49 SD 2,83	3,75E+06 SD 6,58E+06	0,4	7,6E+10 SD 2,15E+10	0,0025
NFKB2	0,204777	1,13 SD 1,92	1,25E+07 SD 1,47E+07	0,27	1,57E+07 SD 1E+06	3,33
TNF α	0,083287	0,95 SD 1,24	1,31E+07 SD 2,8E+07	0,38	4,78E+07 SD 1,2E+07	40
IFN γ	0,081272	1,9 SD 3,19	1,58E+07 SD 2,9E+07	6180	3,1E+06 SD 7,12E+06	0,0004

Transplantation (II)

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RADIOIMMUNOTHERAPY AND HEMOPOIETIC STEM CELL TRANSPLANTATION IN PATIENTS WITH NON-HODGKIN'S LYMPHOMA (NHL): MULTICENTRE STUDY OF 43 PTS.

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High-dose therapy followed by autologous hematopoietic stem-cell transplantation is a main indication for relapsed or high-risk first remission aggressive nHL (4 pts) and plays a role for relapsed follicular lymphoma. Preliminary studies suggest that Y-90 ibritumonab tiuxetan in combination with high dose therapy and autologous stem transplantation is associated with high response rates, durable remissions and acceptable toxicity. We evaluated the combination of Y-90 ibritumonab tiuxetan at standard dose (0.4 mCi/Kg) plus high dose regimen (BEAM or BEAM like) in 40/43 evaluable pts with advanced stage nHL who failed to achieve complete remission (CR) after first line chemotherapy or very high risk pts in 1st CR between April 2006 and May 2008. *Methods.* The treatment plan is shown in Figure 1. PBSCs were collected after mobilization with DHAP and G-CSF. Pts characteristics are shown in Table 1.

Table 1. Patients' characteristics.

	Data
Median age, y (range)	53 (18-74)
Histology, no. (%)	
Follicular	13 (32,5)
Aggressive	27 (67,5)
III-IV stage at diagnosis, no. (%)	31 (77,5)
Median number of prior chemotherapy, no. (range)	2 (2-5)
IPI, grade	
0-I	18 (45)
II-III	22 (55)
Bone marrow involvement at diagnosis, no. (%)	16 (40)
Prior rituximab, no. (%)	36 (90)
Status at transplant	
Progression	8 (20)
PR	20 (50)
CR	12 (30)
Median time to HST, months (range)	15 (5-78)

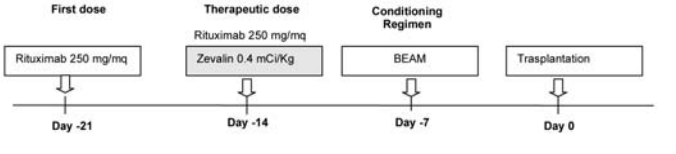

Figure 1. Treatment plan.

Results. The median CD34+ cells infused was 5.5×10⁶/Kg (range 2.5-34). All pts engrafted. The median time to platelet counts higher than 20×10⁹/L were 14 days (range, 9-50 days). The median time to an absolute neutrophil count greater than 0.5×10⁹/L were 10 days (range, 8-20). The median numbers of red blood cells and platelets transfusion were 4 (1-7) and 6 (1-8). Median CD3+, CD4+, CD8+ and CD56+ cells count at day +30 were 700 xmille/mmc (22-3870), 214 xmille/mmc (30-1089), 550 xmille/mmc (40-3090) and 98 xmille/mmc (20-712), respectively. 21 grade III-IV mucositis was documented. Febrile neutropenia occurred in 75% of cases. Median time onset and the duration of fever were day +3 (range, 1-16) and 3 days (range, 1-10). We observed 13 cases of FUO, 8 pneumonitis and 8 blood stream infections, (5 by Gram+). One patient developed an atrial fibrillation. TRM was 5% (septic shock, viral encephalitis) 1 pts died for ARDS at day +230. Median follow-up is 270 days (range 6-690). The EFS 2y is 65% with 24 pts (60%) in CCR. 13/20 pts with PR had a CR after transplant. 3 pts relapsed (11%) and 4 progressed at median day +112 (60-307). 8 pts died, 5 pts for desease progression. C*onclusion*. The use of RIT plus BEAM in relapsed or high-risk first remission aggressive nHL provides good results (CCR 60%) with sustained engraftment, an acceptable extra-haematological toxicity and a rapid immunological recovery. The EFS at 2y is 65%. The power of this program needs to be assessed in a larger series of patients in a randomized trial.

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SUICIDE GENE TRANSFER INTO DONOR LYMPHOCYTES ALLOWS EARLY AND EFFECTIVE IMMUNE-RECONSTITUTION AFTER FAMILY HAPLOIDENTICAL HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR LEUKEMIAS: RESULTS OF THE TK007 STUDY

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Haploidentical family donors represent the ideal solution to offer to every patient with high risk leukemia the potential cure of hematopoietic stem cell transplantation. Extensive application of haploidentical transplantation (haplo-SCT) is limited by high rate of late transplant related mortality (TRM) and relapse associated with the delayed immune reconstitution (IR) secondary to the procedures for severe graft-vs-host-disease (GvHD) prevention. In a haplo-SCT phase I-II multicenter, open, non-randomized trial sponsored by MolMed SpA, we infused donor lymphocytes genetically engineered to express the suicide gene herpes simplex thymidine kinase (TK-DLI) to induce early IR, while selectively controlling GvHD. We enrolled 51 patients (pts) -median age 48- with high-risk hematologic malignancies. Twentynine patients were in remission at transplantation. After myeloablative conditioning regimen, 48 pts received a median 13×10⁶/kg CD34+ and 1.0×10⁴/kg CD3+ (median time to engraftment: 2 weeks). No IR were observed in absence of TK-DLI. Twenty-seven pts received TK-DLI: 22 pts obtained prompt IR with CD3+>100/mcl at day+75 (median) from haplo-SCT and day+23 from TK-DLI. Eleven pts developed GvHD (10 acute GvHD grade I-IV and 1 chronic GvHD) that was always abrogated by the suicide gene induction. The median follow-up is 197 days. The 1-year TRM in intention-to-treat (ITT) analyses was 40% (last event at d+166). The cumulative infectious mortality was 10% in TK-treated immune-reconstituted patients. Immunoreconstitution obtained with TK-cells infusion correlated with rapid development of a wide T-cell repertoire and detection of high frequencies of T-cells specific for opportunistic pathogens. In ITT, the median leukemia-free survival (LFS) for patients transplanted in persistence of disease was at d+169, while patients in remission at the time of transplantation showed an overall survival in ITT of 51% at 1 year. This strategy is feasible and effective in providing IR in haplo T-cell-depleted setting. In uni- and multi-variate analysis both status at transplant and IR are significant risk factor.Infusion of TK-cells could significantly extend the application of haplo-SCT. A randomized phase III study for patients with high risk acute leukemia in remission at SCT has started.

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GEMTUZUMAB-OZOGAMICIN AS MAINTENANCE THERAPY AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION IN ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA

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The role of SCT in elderly patients with AML is under investigation. GO is a recombinant humanized monoclonal antibody conjugated to the antitumour antibiotic calicheamicin targeting CD33 antigen. We hypothesized that GO could have beneficial effects as maintenance therapy after hematopoietic SCT in high-risk patients. Here we report on 5 (3 males and 2 females; 64,67,67,69,60 years respectively) elderly AML patients in CR who received 4 fractionated doses of GO as maintenance therapy following ASCT. Patient #1 was in 2^ CR following an induction regimen of 2 cycles of fludarabine (Flu) + cytarabine (Ara-C) + idarubicin (Ida). He previously underwent ASCT, using a conditioning regimen of Bu-Cy. Patient #2 was in first CR following an induction regimen of 2 cycles of etoposide + Ara-C + Ida. Patient #3 and patients #4 were in first CR following an induction regimen of 2 cycles of Flu + Ara-C. Patient #4 is still in maintenance therapy and, at that time, received two doses of GO. Patient #5 had an AML refractory to a standard induction chemotherapy (3+7 regimen) and rescued with one MEC regimen. Histocompatible donors were not available for any of the patients. Three months after attaining CR, all 5 patients received a myeloablative conditioning regimen and underwent ASCT. Two months after the complete engraftment, patients initiated treatment with GO. All 5 patients remain alive and in CR at +28, +15, +12, +5, +12 months, respectively, while the overall survival at the time of reporting is +51, +20, +17, +9, +21 months. There were no cases of grade 3 or 4 liver toxicity and bleeding was not observed in any of the patients.

Table 1. Disease ouctomes and details of hematological adverse events.

	Patient 1	*Patient 2*	*Patient 3*	*Patient 4**	*Patient 5*
Treatment details					
Period from CR to ASCT	3 months	3 months	3 months	3 months	3 months
Conditioning regimen	BCNU+ Vepesid+ Cytarabine	Busulphan+ Cyclophosphamide	Busulphan+ Cyclophosphamide	Busulphan+ Melphalan	TBI+ Mephalan
Period from ASCT to administration of GO	2 months	2 months	2 months	2months	2 months
GO schedule	6 mg/m² × 2 every 28 days then 3 mg/m² × 2 every 28 days	3 mg/m² × 4 every 28 days	3 mg/m² × 4 every 28 days	3 mg/m² × 4 every 28 days	3 mg/m² × 4 every 28 days
Disease outcomes					
Overall survival	+51 months	+20 months	+17 months	+9 months	+21 months
Continuing in CR	+28 months	+15 months	+12 months	+5 months	+12 months
Hematological toxicity					
Thrombocytopenia (50,000-1000,000/μL)	Yes	Yes	Yes	Yes	Yes
Neutropenia (500-1,000/μL)	Yes	Yes	No	No	No

*CR: complete remission; ASCT: autologous stem cell transplantation; GO: gemtuzumab ozogamicin; TBI: total body irradiation. *At that time, the patient received two doses of GO.*

Thrombocytopenia (50,000-100,000 cells/microL) occurred in all five patients and neutropenia (500-1000 cells/microL) in 2 patients. In conclusion, in our series, despite limited in number, GO demonstrated good efficacy when administered in fractionated doses as maintenance therapy after ASCT in older patients with CD33+ AML in first or second CR. GO showed an acceptable tolerability profile, with no severe hepatotox-

icity and no bleeding. Thrombocytopenia occurred in all five patients and in all cases there was a rapid platelet recovery. A GO dose of 3 mg/m^2 appeared to be better tolerated than the higher dose (6 mg/m^2) and will be used in future studies. Our results could support a new therapeutic role for GO, namely as a maintenance therapy for patients in CR following hematopoietic SCT.

P237

SUBSETS OF CD34+ AND EARLY ENGRAFTMENT IN ALLOGENEIC PERIPHERAL STEM CELL TRANSPLANTATION IN AML PATIENTS

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Engraftment kinetics in allogeneic peripheral blood stem cell transplantation (alloPBSCT) depend on the number and efficiency of the stem cells in the graft, the conditioning regimen and GvHD prophylaxis. Currently, stem cell evaluation is performed by counting CD34$^+$ cells; however, CD34$^+$ cells are a heterogeneous population including the early uncommitted fraction as well as different subsets committed to one or the another lineage; hence, defining the CD34$^+$ subset most predictive of engraftment and its threshold value would be of the utmost importance. This study aimed to identify which graft product subset of CD34$^+$ cells might be the most predictive of early hematopoietic recovery following alloPBSCT .The relationships between the number of "mature" subsets of CD34$^+$ cells (CD34$^+$/CD33$^+$, CD34$^+$/CD38$^+$, CD34$^+$/DR$^+$ and CD34$^+$/CD133$^-$) and "immature" subsets of CD34$^+$ cells (CD34$^+$/CD33$^-$ CD34$^+$/CD38$^-$, CD34$^+$/DR- and CD34$^+$/CD133$^+$) and early neutrophil and platelet engraftment were studied in a homogeneous series (for disease, pre-transplant chemotherapy, conditioning regimen GvHD prophylaxis) of 31 acute myeloid leukemia (AML) patients after alloPBSCT from HLA-identical siblings. All patients received the BU-CY regimen consisting of busulfan 4 mg/kg/day for 4 consecutive days followed by cyclophosphamide 60 mg/kg/day for 2 consecutive days; GvHD prophylaxis included cyclosporin and methotrexate. The CD34$^+$ dose infused ranged from 2.9 to 8.8×10^6/Kg (median 4.6); the percentage of immature CD34$^+$ cells was 40% for CD34$^+$/CD33$^-$, 65% for CD34$^+$/CD38$^-$, 5% for CD34$^+$/DR$^-$ and 70% for CD34$^+$/CD133$^+$; this translates into a median dose of 1.8×10^6/Kg (range 0.3-5) for CD34$^+$/CD33$^-$, 2.8×10^6/Kg (range 0.1-6.2) for CD34$^+$/CD38$^-$, 0.3×10^6/Kg (range 0.05-2.3) for CD34$^+$/DR- and 3.2×10^6/Kg (range 0.6-5.2)×10^6/Kg for CD34$^+$/CD133$^+$. Median time to achieve engraftment of neutrophils and platelets was 13 days (range 10-16) and 15 days (range 13-19), respectively. In our experience the total CD34$^+$/CD133$^+$ cell number was inversely correlated with the days required for recovery of 0.5 ×10^9/L neutrophils (r=-0.82, p=0.02) and 20×10^9/L platelets (r=-0.60, p=0.06); this correlation was better than the total CD34$^+$ cells dose and neutrophil (r =-0.70, p=0.04) and platelets engraftment (r=-0.56, p=0.07). No correlation was found between the other CD34$^+$ subsets and neutrophil and platelets engraftment. We suggest that a high number of CD34$^+$/CD133$^+$ peripheral blood stem cells may be associated with faster neutrophils and platelets recovery; these findings may help to predict the repopulating capacity of PBSC in patients after allogeneic PBSCT, especially when a relatively low number of CD34$^+$ cells is infused.

P238

IN VITRO CHEMOSENSITIVITY OF "NON LEUKEMIC" CFU-GM, IN AML PATIENTS, IS CORRELATED WITH CD34+ MOBILIZATION AND CAN IDENTIFY GROUPS WITH DIFFERENT RELAPSE RISK

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Background. An high number of CD34$^+$ cells in P.B. during mobilization has been associated in AML patients in CR to a high relapse rate and to greater amount of minimal residual disease (Keating, Feller 2003). A different pharmacokinetics of chemotherapy drugs administered during induction or an intrinsic chemoresistence of normal bone marrow precursors have been hypothesized as possible explanations for the observed association between mobilization of non leukemic CD34$^+$ cells and leukaemia residual disease. *Methods.* With this background we assessed in a group of AML in CR the *in vitro* chemosensitivity of non leukemic BM cells to Maphosphamide and Etoposide and correlated it to mobilization strength as well as to DFS. 37 patients affected by AML have been prospectively studied, all were treated using a same induction and consolidation chemotherapeutic regimen. Sensitivity to Maphosphamide and to Etoposide of CFU-GM, BFU-E, CFU-E, CFU-GEMM obtained from bone marrow in 1st CR was studied 2-4 weeks after PBSC mobilization. *Results.* Chemosensitivity of CFU-GM to ASTA-Z as well as to Etoposide and of CFU-GEMM to ASTA-Z, expressed as residual colony growth in comparison to untreated cells, was significantly correlated with peak of CD34$^+$ cells in P.B. during mobilization (R=0.639, p=0.0001 at 75 mcg/mL of ASTA-Z. In univariate and multivariate analysis factors important for failure of CD34$^+$ mobilization were Chemosensitivity to ASTA Z and a PLT count <140×10^9/L. To study relationship between chemosensitivity of non leukemic CFU-GM and survival we splitted patients in the 3 groups according to their chemosensitivity in respect to normal controls. Survival at 6 months was 0% for hyposensitive group, 60% in normosensitive group and 78% in hypersensitive group (log rank: 0.064). The survival as well as the relapse incidence of hyposensitive patients was significantly lower than the remaining patients (log rank: 0.01) (Figure 1), moreover ASTA-Z sensitivity of normal non leukemic CFU-GM was found important for OS and Relapse Incidence also in group of AML pts having at diagnosis a normal cytogenetic. *Conclusion.* We have found that in AML patients sensitivity of normal non leukemic CFU-GM to maphosphamide and to Etoposide is highly variable and significantly correlated to CD34$^+$ cells peak reached during mobilization. Chemosensitivity of normal non leukemic CFU-GM was also found to be related to prognosis of the disease.

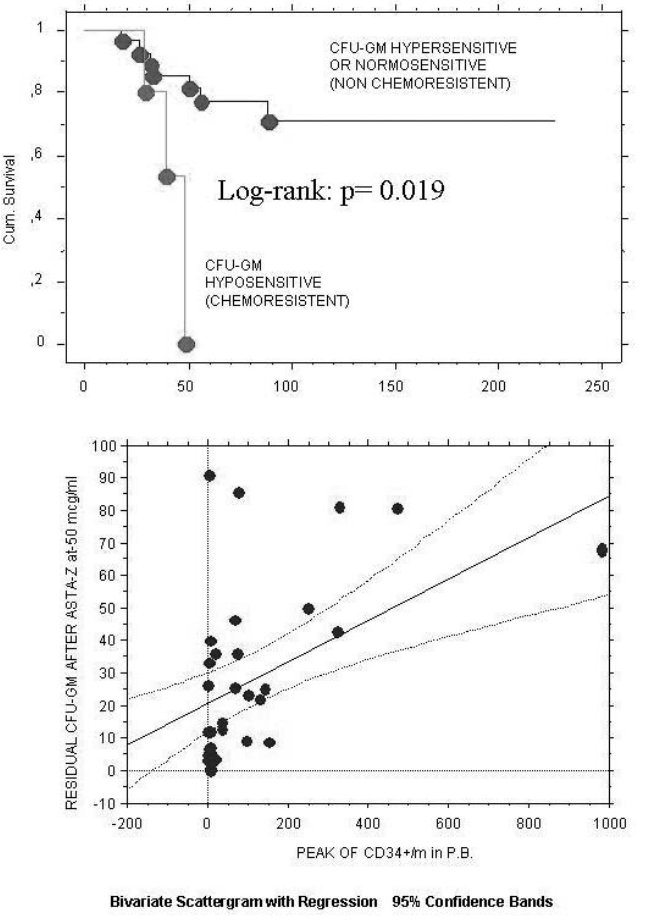

Figure 1. Kaplan-Meier Cum. Survival Plot for DFS in groups defined according sensitivity of CFU-GM TO ASTA-Z.

P239

CONTINUOUS INFUSION HIGH DOSE IDARUBICIN AND INTRAVENOUS BUSULPHAN AS CONDITIONING REGIMEN TO AUTOLOGOUS STEM CELL TRANSPLANTATION IN PATIENTS WITH ACUTE MYELOID LEUKEMIA

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A tentative approach for reducing relapse rate in AML is the adoption of specifically designed conditioning regimens. We previously reported encouraging data using IBU regimen, based on combination of idarubicin (IDA) and oral busulphan (BU). While results in terms of relapse rate were promising, most relevant toxicity was oral mucositis, recorded in more than 90% of patients requiring in most cases total parenteral nutrition (TPN). Here we describe preliminary results achieved in 16 patients with a new regimen based on replacement of oral with intravenous BU. The protocol included IDA at 20 mg/sqm daily as 3 days continuous infusion (from day -13 to -11) and intravenous BU at 3 mg/kg daily from day -5 to -2. Patients aged over 60 years (n=6) received a reduced schedule (two days IDA and 3 days BU at the same dose). The median age was 51 years (30-72). All patients received peripheral blood stem cells. The median interval between diagnosis and ASCT was 4 months (3-7). The median number of $CD34^+$ cells infused was $6,9\times10^6$/kg (3.1-18). All patients were autografted in conventional single bed rooms. The median number of days to PMN >500/cmm and platelets >20000/cmm was 11 (8-13) and 13 (8-40), respectively. The median number of platelet and blood units transfused was 3 (1-6) and 2 (0-4), respectively. No grade 3 or 4 episode was recorded. In particular, grade 3-4 mucositis was absent and no patient required TPN. Eight patients experienced FUO, while fever did not occur in 8 patients at all. As compared to previous series, the occurrence of severe mucositis was dramatically reduced (88% *vs.* 0%, $p<0.0001$). In addition, the incidence of fever (p:0.01), documented infections (p:0.03) and need and duration of i.v. antibiotic therapy were also significantly reduced. No transplant related death occurred. Median time of hospitalization was 27 days (20-37) and it is shorter as compared to oral BU (p:0.01). At the time of writing, 5 patients have relapsed and 11 are alive in continuous CR with a median follow up after ASCT of 10 months. We conclude that replacement of oral with intravenous BU results in a more favorable toxicity profile. The incidence of mucositis is significantly reduced with relevant decrease in TPN, antibiotics needing and hospitalization. A longer follow-up is required to assess a potential advantage in terms of disease free survival. Intravenous Bu should replace the oral formulation in each regimens adopting BU as conditioning to ASCT.

P240

KINETIC OF HEMATOLOGICAL TOXICITIES IN PATIENTS TREATED WITH RADIOIMMUNOTHERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATIONFOR NON-HODGKIN'S LYMPHOMA

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Background. Neutropenia and thrombocytopenia are the most common hematological toxicities in relapsed-refractory NHL pts treated with RIT (Zevalin®). About 50% of pts experienced grade 3-4 neutropenia (ANC $<1\times10^9$/L) and 60% grade 3-4 thrombocytopenia (PLT $< 50\times10^9$/L). The nadir for neutropenia and thrombocytopenia is around day +60 after Zevalin® treatment without stem cell rescue (1-2). *Aims*. Poor informations exist about kinetic of late hematological toxicities in pts treated with RIT included in a high dose chemotherapy program (Z-BEAM). *Methods*. We evaluated kinetic of hematological toxicities in 14 high risk NHL pts, treated with radio-immunotherapy followed by high dose chemotherapy (Z-BEAM) and ASCT from February 06 to May 2008. RESULTS: At transplant 3/14 pts were in 1st complete remission (CR), 7/14 in partial remission (PR), 2 pts were in relapse (REL) and 2 in progression (PROG). Median CD 34^+ cells infused was $4,07\times10^6$/Kg (range 2,66-21,6). All pts engrafted. Median time to ANC $\geq0,5\times10^9$/L was 11 days, median time to platelets (plt)$\geq20\times10^9$/L was 13 days. After hematological reconstitution 5/14 pts experienced grade 3-4 neutropenia (range $0.33\text{-}0.98\times10^9$/L). Median time of onset was day + 84 (range 32-147). ANC $\geq1\times10^9$/L was achieved at median day +152 (range 35-175). 4/14 pts showed grade 3-4 thrombocytopenia. Median time of onset was day +58 (range 34-291). and 1 pt never achieved plt $\geq50\times10^9$/L. Three pts died with plt $<10\times10^9$/L for BK virus encephalitis at day +34, hyperosmotic coma at day +55 and hemorrhagic shock at day +35. 3 CMV reactivation, 3 urine infection. 2 blood colture + for bacteria and 1 pneumonia was demonstrated during neutropenia and thrombocytopenia (Table 1). *Conclusions.* In our cohort of 14 pts treated with Z-BEAM + ASCT after normal engraftment 5 pts showed grade 3-4 neutropenia and thrombocytopenia. Time of neutropenia onset in this subset of pts seems to arise later (g+84) than in pts treated with Zevalin® alone. Z-BEAM and ASCT is feasible but warning is needed regarding possible late hematological toxicities and the risk of late infectious complications.

References

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2. Witzig JCO 2003;21:1263-1270.

Table 1.

	Age	Sex	Histology	BOM at diagnosis	Status pre SCT	Date Zevalin	CD 34+ infused	1st day Plt <50 (10^9/L)	1st day Plt >50 (10^9/L)	1st day ANC <1(10^9/L)	1st day ANC >1(10^9/L)	Note (During neutropenia o thrombocytopenia)
1	N.L.	69	f	MCL	-	PR	21.7.06	6,8	5 g+43	not achieved	-	-	Hyperosmotic coma exitus g+55
2	F.M.	69	f	DLBCL	+	PROG	28.7.06	3,4	6 g+34	not achieved	-	-	Encephalitis exitus g+34
3	A.C.	57	f	DLBCL	+	PR	7.12.06	3,5	40 g+291	55 g+300	-	-	Urine + (Enteroc.faecium) blood colture:+ (Stph.epidermidis) pneumonia
4	C.R.	37	f	CCCB	-	CR	27.4.07	3,3	-	-	0,980 g+104	1,630 g+152	Urine + (E.Coli)
5	P.A.	68	m	DLBCL	-	CR	8.6.07	4,07	-	-	0,620 g+76	1,010 G+163	CMV urine+
6	V.A.	58	m	DLBCL	-	PR	8.9.07	8,9	-		0,890 g+147	1,010 g+175	Urine + (Enteroc.faecium + Morganella m.)
7	V.V.	57	m	MCL	+ (t 11;18)	CR	9.11.07	5,02	42 g+74	95 g+94	0,500 g+84	1,910 g+95	CMV DNA urine+ gargle+ blood colture CVC+: (Stph.epidermidis)
8	C.M.	68	m	FOLL	-	PROG	7.3.08	2,66	not achieved	not achieved	0,330 g+32	2040 g+35	CMV gargle+ hemorrhagic shock exitus +56

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TETRAMER-BASED QUANTIFICATION OF CYTOMEGALOVIRUS (CMV)-SPECIFIC CD4+ AND CD8+ T LYMPHOCYTES AFTER ALLOGENEIC STEM CELL TRANSPLANTATION MAY IDENTIFY PATIENTS AT RISK FOR CMV INFECTION/DISEASE

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Recovery of cytomegalovirus (CMV)-specific T-cells after allogeneic stem cell (SCT) is critical for protection against CMV disease; in humans, both the CMV-specific $CD4^+$ and $CD8^+$ arms of T-cell immune response must be regenerated after SCT in order to obtain long-term protection against CMV reactivation and disease. In our study we used fluorochrome-conjugated tetrameric complexes of HLA-A101, HLA-A201, HLA-B702, HLA-B801, HLA B3501 to monitor recovery of CD4 and CD8 CMV-specific T-cells (according to the patient's HLA) in 47 patients after SCT; the patients were transplanted with unmanipulated peripheral blood stem cells from an HLA identical related donor (n=45) an HLA identical unrelated donor (n=2). To prevent CMV disease all patients received acyclovir 10 mg/Kg intravenously every 8 h until engraftment and then 1200 mg/day p.o. until day +180. Patients were monitored for CMV infection and disease using peripheral blood mononuclear cells obtained weekly until 1 year. Median age was 36 years (range 18-61); diagnoses were acute myeloid leukaemia (n=37), acute lymphoblastic leukaemia (n=5), chronic myeloid leukaemia (n=3), lymphoma (n=1), myelofibrosis (n=1). Five patients (R-) were CMV seronegative and 3 of them received grafts from a CMV-seropositive donor; forty patients (R^+) were CMV seropositive. The median absolute number of CMV-specific $CD4^+$ T-cells detected at 1, 3, 6, 12 months was 1 μL (range 0 -6), 3 μL (range 0-12), 4 μL (range 0-19) and 8 μL (range 0-48), respectively. The median absolute number of CMV-specific $CD8^+$ T-cells detected at 1, 3, 6, 12 months was 2 μL (range 0-15), 16 μL (range 0-22), 21μL (range 0-47), 22 μL (0-62), respectively. Tetramer analysis showed that 27/47 (57%) patients reconstituted CMV-specific $CD4^+$ and $CD8^+$ T-cells at 3 months; in this group only 3/27 (11 %) patient developed CMV infection. CMV infections were observed in 18/20 (90 %) who failed to generate CMV-specific $CD4^+$ and $CD8^+$ T-cells response. In our experience no CMV infection/disease was observed with CMV-specific $CD4^+$ T-cells> 2 μL and CMV-specific $CD8^+$ T-cells> 5 μL. Recovery of both CMV-specific $CD4^+$ and $CD8^+$ T-cell immunity occurred in 40/42 (95%) R^+ patients within 6 months and 3/5 (60%) R- patients within 12 months. The cumulative incidence of CMV infection was 21/47 or 44% at 1 year, with a median reactivation time of 45 days (range 28-96); one patient, without CMV-specific $CD4^+$ and $CD8^+$ T-cells recovery, developed CMV disease (colitis-pancreatitis) and died. In conclusion we suggest that failure to recover CMV-specific $CD4^+$ and $CD8^+$ T-cells after SCT is associated with the development of CMV infection/disease and we envision that this strategy may enable us to identify those patients who may benefit from preemptive therapy, in particular the adoptive transfer of CMV-specific T lymphocytes for the prevention of CMV disease.

P242

COLLECTION OF PERIPHERAL BLOOD PROGENITORS CELLS AFTER INTERMEDIATE DOSE ARA-C IN PREVIOUSLY POOR MOBILIZER PATIENTS WITH HEMATOLOGIC MALIGNANCIES

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Autologous transplantation of PBPCs is the best therapy for patients with relapsed/refractory hematologic malignancies. Several lines of treatment, alchilating agents, cisplatin and fludarabine, are all factors adversely affecting PBPCs mobilitation. In this study we assessed the efficacy and safety of ARA-C intermediate dose given to mobilize PBSCs in 11 patients (5 M; 6 F; median age 59 yrs; range 35-67 yrs) with hematologic malignancies: 4 non-Hodgkin's lymphoma (NHL), 4 multiple myeloma (MM), and 3 acute myeloid leukemia (AML) who have failed a previous attempt between January 2007 and April 2008. Patients were primed using intermediate dose of ARA-C administered intravenously at a dose of 800 mg/m^2 every 12 h for 6 consecutive doses, + rhG-CSF 5 or 10 microg/Kg subcutaneously. A median of 3 chemotheraputic regimens (range 2-6) were previously given and all patients failed prior harvesting of PBSCs. Two patients with MM were given two autotransplants with Melphalan 200 mg/mq as conditioning regimen. Collection of PBSCs was successfull in 8 out of 11 patients (4 NHL and 4 MM). However, all patients with AML failing mobilization. Harvesting of PBSCs was performed at a median time of 9 days (range 8-12 days) after ARA-C administration. The median number of sucutaneous injections of rhG-CSF was 10 (range 8-12). The median number of WBC count was 4100/mmc (range 1700-11400) at the time of collection with $CD34^+$ cell median number of 1.2 % (range 0.5-3) and 59/microL (range 17-135). In all mobilizer patients the required number of $CD34^+$ cells were harvested after a single leukapheresis. The median number of $CD34^+$ cells collected was 4.42×10^6/Kg (range 2.56-17) and the median number of cryopreserved bags was 4 (range 2-8). All patients experienced neutropenia <500/microL, but only 5 out of 11 had febbrile neutropenia (1 to 4 days). Seven patients received a median of 1 packed red cell transfusions (range 1-3) and 9 patients a median of 1 apheretic platelet products (range 1-3). No patients experienced WHO grade III-IV mucositis and diarrhoea. Our experience, despite limited in number, showed that intermediate-dose of ARA-C + rhG-CSF is safe and effective to mobilize $CD34^+$ progenitor cells in patients who have failed a previous attempt. Mobilization and collection of PBPCs was found independent from the number and type of previous chemotherapies. Patients with AML, previously treated with high-dose ARA-C in induction phase, failed remobilization.

Transplantation (III)

P243

TRACKING ANTIVIRAL T CELLS IMMUNE RESPONSE IN HHV8-ASSOCIATED DISEASES BY INNOVATIVE ELISPOT ASSAY USING FULL-LENGTH RECOMBINANT VIRAL PROTEINS

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T cells immune activity against Human Herpesvirus-8 (HHV8), the etiologic agent of Kaposi Sarcoma (KS) and some rare lymphoproliferative disorders (Primary Effusion Lymphoma, PEL or Multicentric Castleman Disease, MCD), has been only partially deciphered. So far, only few viral epitopes have been demonstrated to elicit a virus-specific T cell response. Moreover, all reports tracking the specific antiviral immunity against single short immunogenic peptides, in different KS clinical settings and HHV8$^+$ healthy subjects, are only referred to HLA-A2$^+$ individuals. The aim of this study is to evaluate the clinical significance of anti-HHV8 T cell immune response in 31 patients with KS, PEL or MCD and in 3 HHV8$^+$ subjects without disease, by innovative application of IFNg-ELISPOT assay using two recombinant viral proteins (orf73, orfK8.1), as common antigenic stimulation for all patients irrespectively to their HLA. In our KS series, antiviral T cells responses were virtually absent in all patients, at the time of diagnosis. Conversely, at the time of disease remission, and in healthy HHV8$^+$ patients, consistent T cell response was clearly detectable after stimulation with both viral antigens. Interestingly, we identified different thresholds defining protective levels of T cell response, between diverse HHV8$^+$ subsets (p<0.001); at the time of KS remission, the lower medium level of T cell response in post-transplant/iatrogenic KS patients (81 SFC/106PBMCs), in comparison with classic KS subset (182 SFC/106PBMCs) would suggest some different protective potentials of T cells in the two clinical forms of KS. No significant correlation was shown between HHV8 viremia and clinical course. Of note, in comparison with KS, PEL and MCD patients showed a less clear-cut correlation between antiviral T cell response and disease course. In conclusion, here we show an IFNg-ELISPOT assay, using recombinant viral proteins, which is suitable for monitoring anti-HHV8 T cell response in patients with different HLA. Our results support the critical role of anti-HHV8 immune surveillance in KS onset and regression, in all its clinical forms; however, anti-HHV8 T cell responses showed variable magnitude between different KS settings, which is likely to depend on immunological conditions underlying the development of HHV8 related disorders. Our study provides further immunological data encouraging the exploration of adoptive cell therapy approaches for treatment of the HHV8 driven neoplasia.

P244

PROSPECTIVE MULTICENTER TRIAL OF REPEATED COURSES OF BONE MARROW-DERIVED CELL MOBILIZATION INDUCED BY GRANULOCYTE-COLONY STIMULATING FACTOR IN AMYOTROPHIC LATERAL SCLEROSIS: FEASIBILITY, SAFETY AND EXTENT OF PROGENITOR CELL MOBILIZATION

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Background. Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder, characterized by the progressive loss of cortical and spinal motor neurons and an outcome usually fatal within 3 to 5 yrs, with no effective therapies presently available. Efforts are needed to find novel treatments. In this view, recent studies have raised the interest for the use of bone marrow-derived cells (BMCs) in order to reduce neuronal loss and/or to rebuild damaged neuronal circuits. Aims of the study. A multicenter trial has been performed to verify possible benefits of repeated procedures of G-CSF-induced BMC mobilization in ALS patients. Rate of BMCs mobilization and main toxic events observed in the trial are here reported. Patients and methods. Six Neurology Centers, along with six Hematology Centers, participated to the "STEMALS" trial. Trial design included 4 mobilization procedures every 3 months. BMC mobilization was induced by G-CSF administered at the dose of 5 µg/kg s.c., twice a day, for 4 consecutive days. At each cycle, BMC mobilization was monitored by daily evaluating both CD34$^+$ve and clonogenic cells, since day 0 through day 6. The trial started in June 2006 and 23 ALS patients have been enrolled. Their median age was 57 yrs (range 40-64), 12 were male. *Results.* Overall, data have been collected on 15 patients who completed the four-cycle schedule plus 5 patients who received the initial two G-CSF courses. BMC mobilization was obtained in all patients, at every G-CSF course, with peak values recorded between day 3 and 4 since G-CSF start. As show in the Figure, median peak values of circulating CD34$^+$ve cells/µL were 57, 57.2, 46.9 and 41, at the 1st, 2nd 3rd and 4th mobilization course, respectively, without statistically significant differences. A parallel increase of circulating myeloid and erythroid clonogenic cells was recorded as well. Overall, the mobilization procedures were well tolerated, with the exception of a transient increase of growth hormone level in one patient, and a deep venous thrombosis in one patient, both complications did not preclude to conclude the treatment. *Conclusion.* The STEMALS trial indicates that: i. the use of G-CSF to induce BMC mobilization is well tolerated and feasible in ALS; ii. BMC mobilization capacity in ALS is analogous to that commonly observed in the healthy population; iii. there are no signs of impaired mobilization, after repeated courses of G-CSF administration, performed at few month intervals.

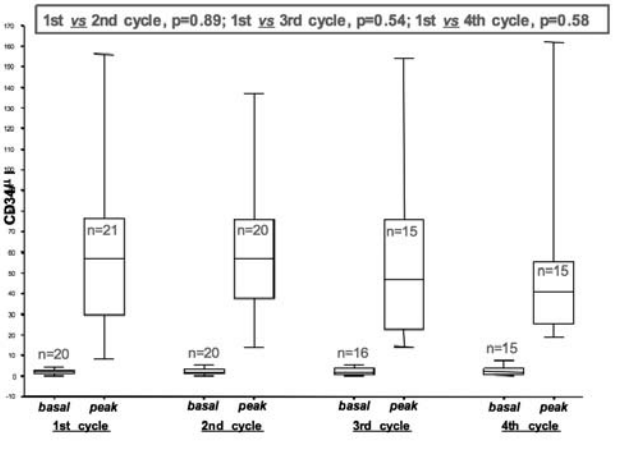

Figure . 1 Box plots rapresent maximum (top), minimum (bottom), quartiles (white box) and median values of CD34+ve cells per µL, at day 0 (basal) and at peak time, in ALS patients, treated with four cycles of G-CSF

P245

ROLE OF GRAFTED CELLS IN DETERMINING THE LENGTH OF TELOMERE OF BONE MARROW HEMATOPOIETIC CELLS FOLLOWING AUTOLOGOUS OR ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Telomeres are DNA sequences located at the end of chromosomes. In somatic cells telomere length (TL) decreases at each cell division and can be considered as a marker of cell aging. In a previous study we have analysed TL in mobilized peripheral blood stem cells (PBSC) collected for autograft. TL was found markedly influenced by the number of high-dose (hd) chemotherapy courses delivered prior to collection; the rate of telomere shortening in PBSC was also found to influence post-autograft TL. *Aim of the present study*. To verify the role of grafted cells in determining the length of telomere in bone marrow (BM) cells from patients undergoing either autologous or allogeneic hematopoietic stem cell transplantation (HSCT). *Patients and Methods.* TL analysis was performed on cells obtained from 20 patients following PBSC autograft and 10 patients following allogeneic HSCT. They all were in Con-

tinuous Complete Remission following HSCT, with Cell Blood Counts within normal ranges. Median follow-up since HSCT was 18 months. Patients of the autograft group had received a hd-schedule including hd-cyclophosphamide (CY) and hd-Ara-C. All patients were grafted with large amounts of PBSC, with a median of 7×10^6 grafted CD34+ve/Kg cells (range 3,27–22,6). TL assay was performed using Southern Blot. *Results.* In the autograft group, TL was markedly reduced in PBSC collected at the second hd-course (hd-Ara-C) compared to PBSC post hd-CY. TL was then evaluated following HSCT in 10 patients autografted with post-CY PBSC and 10 with post-Ara-C PBSC. As shown in Figure 1A and B, patients that received post-Ara-C PBSC had BM TL (7120 bp) that corresponded to the TL of grafted cells; patients that received post-CY PBSC had significantly longer BM TL (7614 bp) compared to pre-transplant TL ($p<0.05$); in this subgroup, post-HSCT TL was analogous to that of post-CY PBSC. Finally, TL was studied in the allograft setting (Figure 1C). Donor PBSC showed longer TL than recipient BM; after allografting, these patients displayed a median TL (7381 bp) analogous to the donor PBSC TL (7113 bp) and longer than their pre-allograft TL (5959 bp). *Conclusion.* i. TL of post-HSCT BM cells reflects that of grafted cells; ii. TL of grafted cells, rather than replicative stress of engraftment, influenced telomere state following HSCT; iii. the use of younger cells is advisable for grafting in order to maintain adequate TL post-HSCT.

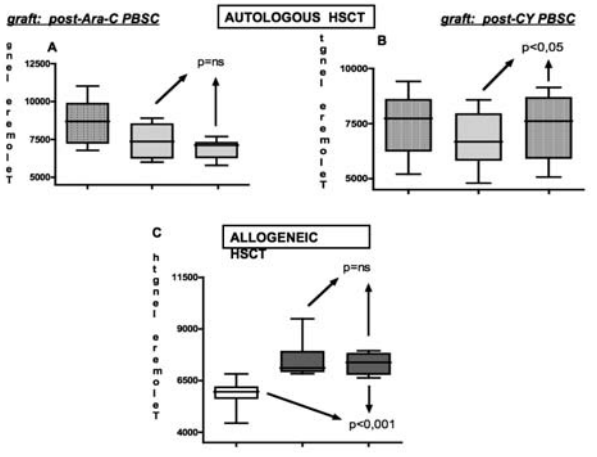

Figure 1.

P246

ATROGENIC LYMPHOCYTE ABLATION AND VIRAL REACTIVATION: EFFECTIVE PREVENTION BY PROPHYLACTIC VALGANCICLOVIR

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Intensive immunosuppression (IIS) by lymphocyte-depleting agents may be hampered by the infectious risk associated with long-lasting lymphocytopenia; this concerns CMV and other long-latency viruses, such as EBV, HSV, VZV, but also HBV and HCV. Alemtuzumab (ALE) is an anti-CD52 mAb with powerful lymphocytolytic activity potentially effective in immune-mediated marrow failure syndromes. We investigated anti-viral prophylaxis in a cohort of 20 patients suffering from aplastic anemia (SAA, n=7), pure red cell aplasia (PRCA, n=10) or agranulocytosis (AGR, n=3) receiving subcutaneous ALE as IIS (4-5 day course, total dose 73-103 mg). Anti-viral prophylaxis, mostly directed against CMV, was carried out by oral valganciclovir (VAL) 450 mg twice daily, administered to all seropositive patients; CMV antigenemia was monitored weekly by PCR (detection limit 1000 copies/mL). A single SAA patient with occult HBV ($HBcAb^+$) also received lamivudine. All patients showed complete lympho-ablation within 2-3 days, which lasted several months; VAL was administered until circulating $CD4^+$ T cells reached 100/L, or for a maximum of 3 months. No hematological toxicity was observed. At a median follow-up of 12 months, infectious events were irrelevant: one patient developed early (day +2) HSV of the upper labium, not requiring additional therapy; no patient developed CMV disease, EBV-related diseases or even fever or lymphoadenopathy. At 6 months from treatment (3 months after discontinuation of VAL), one patient developed a VZV reactivation with shingles (L5 dermatome), requiring specific treatment (valaciclovir). Some patients required further ALE (either as single doses or complete courses) due to relapse of their hematological disease; they did not receive additional prophylactic VAL. Three of them showed CMV reactivation just above the detection limit of the PCR; VAL was administered as pre-emptive treatment, leading to prompt clearance of the viremia. One AGR patient with anti-HBcAb IgG developed frank HBV reactivation with seroconversion; lamivudine was promptly administered and resulted in viral load drop in absence of any laboratory sign of hepatitis. In conclusion, viral complications in bone marrow failure patients may be effectively prevented by oral VAL, even in presence of extreme and long-lasting lymphocytopenia. The optimal treatment duration, as well as the need for additional agents targeting other viruses, namely HBC or HCV, will deserve further investigation.

P247

T-CELL CHIMERISM AND CLINICAL OUTCOME AFTER REDUCED-INTENSITY CONDITIONING ALLOGENEIC STEM CELL TRANSPLANTATION

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Objectives. This study investigated the kinetics of whole peripheral blood (PB) and $CD3^+$ T-cells chimerism in patients receiving reduced-intensity conditioning (RIC) allogeneic stem cell transplantation (SCT). The T-cell chimerism has been correlated with risk of grade II-IV acute graft-versus host disease (GvHD) and relapse. *Materials and methods.* Twenty-one patients with a median age of 54 years (range 17-65) affected by lymphoma (13), multiple myeloma (5), acute myeloid leukaemia (1), acute lymphoid leukaemia (1) or idiopathic myelofibrosis (1) received RIC allogeneic SCT at Hematology Division of Udine between January 2007 and February 2008. Source of stem cell was PB and donors were matched unrelated for 14/21 patients. Conditioning regimens were: thiotepa plus cyclophosphamide (15), 2 Gy total body irradiation (TBI) plus fludarabine (3), melphalan plus fludarabine (3). In 16 cases anti-thymocyte globulin was used as part of GvHD prophylaxis. Hematopoietic chimerism has been serially assessed at 30, 60, 90 and 180 days after SCT in whole peripheral blood (PB) and sorted $CD3^+$ T-cells. The analyses have been performed by polymerase chain reaction (PCR) based amplification of short tandem repeats (STR) sequences using the AmpflSTR identifler kit (Applied Biosystems). Full donor chimerism (FDC) was defined as the presence of at least 95% donor cells. Results. The percentage of patients achieving FDC was lower in sorted CD3+ cells in comparison with whole PB at day 30 (75% *vs.* 80%) and 60 (70% *vs.* 80%), but the difference was not statistically significant. At days 90 and 180 the percentage of patients achieving FDC was similar in CD3+ cells and PB. Patients with grade II-IV acute GVHD had no significant difference in the incidence of $CD3^+$ FDC at day 30, 60, 90 after SCT in comparison with patients with≤ grade I GVHD. However, patients who subsequently relapsed had a significant lower incidence of $CD3^+$ FDC at day 60 (20% *vs.* 87%, $p=0.005$) and at day 90 (50% *vs.* 92%, $p=0.06$) in comparison with patients with sustained remission. Conclusions. We conclude that the T-cell populations reached FDC more slowly than myeloid compartment in RIC-SCT. Moreover, mixed chimerism at days 60 and 90 was associated with an increased risk of relapse. The development of acute GvHD was apparently not correlated with T-cell chimerism.

P248

IMPACT OF QUALITY CONTROL POLICY ON UNRELATED CORD BLOOD TRANSPLANT

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Cord Blood Units (CBU) are increasingly used for unrelated allogeneic stem cell transplantation and their selection is meanly done according to the nucleated cell (NC) dose and HLA donor/recipient compatibility. Objectives: The aim of our study was to evaluate the impact of quality control (QC), performed before CBUs formal recruitment, on

post-transplant outcome. Methods: We retrospectively analyzed 42 unrelated CBT performed with 46 CBU (4 double transplants) at Rome Transplant Network (RTN) since September 2005 to March 2008. Patients underwent unrelated CBT for malignant (n= 39) or non malignant diseases (n= 3) either in 1st/2nd complete remission (n=17/8) or in advanced disease (n= 17). Median patient age and body weight were 18.2 years (0.6-60) and 50 kg (4 -96), respectively. The CBU were HLA matched in 3 cases and mismatched for 1 (n=10) or 2 HLA loci (n=33) in 43 cases. According to RTN policy, CBU selection was based on the following pre-freezing parameters: NC dose >2.5×10^7/kg, HLA compatibility >4/6 loci, $CD34^+$ cell count > 1×10^5/kg and CFU-GM > 1×10^4/kg and AB0 compatibility. In order to confirm the reproducibility of pre-freezing data, the cell recovery in terms of NC, $CD34^+$ cell count, clonogenic assay and viability was required after thawing of an aliquot before the formal recruitment of CBU for transplant; confirmatory CBUs and mother HLA typing, sterility after thawing and lacking maternal virology tests were also evaluated Results No significant difference was observed between QC and pre-freezing/infused cell dose in terms of $CD34^+$ and CFU-GM, otherwise there was a significant statistical difference between QC and infused NC. The median time between CBU freezing and thawing was 5.2 years (0.26-9.04) and did not affect the $CD34^+$ and CFU-GM recovery. The $CD34^+$ cell count evaluated at QC resulted significantly related to the a-GVHD development and the viability of CBU at QC and infusion significantly correlated with speed of engraftment for both PMN (p=0.04) and PLTS (p=0.005). Conclusions: From this study we can draw the following preliminary conclusions: 1) the viability of cells is related to the speed of engraftment; 2) there is a relationship between QC as $CD34^+$ count and occurrence of aGVHD; 3) QC are predictive for the transplant outcome and reflects the safety of cryopreservation procedure.

P249

EXTRAMEDULLARY BONE RELAPSE AFTER ASCT OF MEGAKARYOBLASTIC LEUKEMIA IN A CHILD WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA

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A 18-month child came to our department for isolated thrombocytopenia, dating from birth; platelet (Plt) had been about 50.000/microL; antiplatelet antibodies were not detectable, immunological tests and abdominal ultrasonography were negative. Bone marrow aspiration (BMA) showed an increased megakaryocytopoiesis. It was diagnosed as idiopathic thrombocytopenic purpura (ITP), without bleeding manifestations, no therapy being necessary. After 10 months the thrombocytopenia worsened (Plt 20.000/microL) but the BMA excluded acute leukemia. After 4 weeks, when Plt reduced to 7000/microL and anemia first occurred, the peripheral blood (PB) and BMA showed 20% and 50% of blasts positive for CD33, 38, 41, 61,105 and negative for CD15, 34, 117, MPO; karyotype was normal and so analyses for chromosomal translocations BCR/ABL, AML1/ETO, CBFbeta/MYH11, DEK/CAN, PML-RARalpha and so the liquor examination. BM trephine biopsy confirmed acute megakaryoblastic leukemia (AMKL). The child underwent chemotherapy according to ICE, AVE, HAM protocols and finally allogeneic peripheral stem cell transplantation (ASCT) from MUD. During the follow up only a mild chronic GVHD occurred. After 17 months he presented a swelling at right humerus; the diagnostic imaging showed an extensive signal alteration pattern suggesting a malignant infiltration. Histological examination of the biopsied bone tissue showed a proliferation (MIB1++) of immature leukemia cells, positive for LAT and CD41, negative for CD20, 3, 30, 117, 99, cytokeratin, CD68/PG-M1. The PB smear and BMA showed no evidence of relapse; almost complete donor chimerism was documented. All these features were consistent with granulocytic sarcoma, of megakarioblastic type, extramedullary recurrence of AMKL. Chemotherapy was administered according to MyFLAN plus local irradiation. The child is now undergoing a second allogeneic transplantation with TBI conditioning. AMKL occurs in less than 10% of childhood AML and in our case the BMA, performed during the follow up of the ITP, was not able to rule out its sudden onset. The extramedullary myeloid sarcomas (EMS) are rare, destructive, tumor masses consisting of immature leukemia cells. They have been reported rarely as recurrences of AML after ASCT, particularly in patients with GVHD; usually a subsequent bone marrow relapse follows, unlike our case. EMSs result frequently in treatment failure and in a poor prognosis with conventional therapy, so an aggressive treatment is mandatory.

P250

USE OF PALIFERMIN IN THE PREVENTION OF ORAL MUCOSITIS IN PATIENTS UNDERGOING STEM CELLS TRANSPLANT: A SINGLE CENTRE EXPERIENCE

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Background. Oral Mucositis (OM) is a frequent and discomfortable adverse event in patients receiving high-dose chemotherapy and autologous stem cells transplantation. Moreover the risk of infection is directly related to the severity of OM. Some clinical studies have shown the ability of Palifermin, a recombinant human keratinocyte growth factor (KGF), to decrease the incidence, the grade and the duration and duration of mucositis in transplanted patients.by non Hodgkin's and Hodgkin's Lymphoma. *Patient and Methods.* We analyzed five patients undergoing autologous stem cells transplant in our Institution between May 2007 and May 2008, Palifermin was utilised to prevent OM. Three patients were male and two female, with a median age of 67 ys (range 25-69). They all suffered by non Hodgkin's Lymphomas: two Diffuse Large B cell, one Mantle cell, two Follicular Lymphomas. Performance status according to Karnofsky score was ranged between 80% and 100%. All patients were pretreated by almost two lines of polichemotherapy. High-dose chemotherapy consisted of an association of radio-immunoterapy (Zevalin) with hig-dose chemotherapy regimen BEAM (Z- BEAM) followed by autologous stem cell rescue. Palifermin was administred at the standard dose of 60 micrograms per kilogram of body weight per day intravenously for three consecutive days before the start of conditioning chemotherapy and after stem cells infusion. *Results.* Only one patient (1/5: incidence 20%) developed a grade 3 OM, according to WHO oral-toxicities scale, lasting five days, not requiring opioid analgesics, but only small doses of FANS and total parenteral nutrition (TPN) for five days. Two patients required TPN, for respectively five and seven days, for nausea and anorexia. None of our patients had increased levels of serum lipases and amylases, and only one shown non-itching erythema of face and neck, that lasted two days and not required use of antihistaminic drugs, but leaved cutaneous pigmentation. Median time to hemopoietic reconstitution for ANC ≥0,5 ×10^9/L and platelets (plt) ≥20 ×10^9/L was 10 days (range 9-11). The median duration of febrile neutropenia was 3 days (range 2-10), with only a documented sepsis by Escherichia Coli, Serratia Marcescens and Staphilococcus Epidermidis in one patient. The median duration of hospitalization was 24 days (range 20-37). In a control group of 35 patients that underwent Z-BEAM regimen in the same period without KGF administration the incidence of grade 3-4 OM was 60 %. *Conclusions.* Our experience, according to previously reported data, suggests that use of Palifermin possibly reduce the incidence, the severity and the duration of oral mucositis, with a decreased need of use of TPN and opioid analgesics, and with very poor undesirable effects. This approach can also reduce time of hospital stay and risk of infections in patients undergoing autologous stem cells transplant.

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PUB01

LIPOSOMAL CYTARABINE IN THE CENTRAL NERVOUS SYSTEM PROPHYLAXIS OF PATIENTS WITH NON-HODGKIN'S LYMPHOMA AND LYMPHOBLASTIC ACUTE LEUKEMIA RESULTS OF A SINGLE-CENTRE EXPERIENCE

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Background. CNS involvement In NHL, meningeal relapse occurs more rarely, ranging from 4–14% depending on histology, anatomical location and biological parameters. Flow cytometric analysis of cerebrospinal fluid (CSF), however, detected occult lymphomatous meningitis in 22% of NHL cases at risk at diagnosis. As occult CNS involvement has been shown to occur in >20% of newly diagnosed patients with high-risk NHL, flow cytometry and cytospin analysis of CSF at diagnosis should be implemented in order to adequately target CNS prophylaxis (Hegde *et al.* Blood 2005;105:496). CNS prophylaxis is currently recommended only in high-risk disease (stage IV/high IPI score) and in patients with extra nodal NHL. CNS involvement in acute lymphoblastic leukemia is a well-recognized event and CNS prophylaxis is considered mandatory. Sustained-release liposomal cytarabine (DepoCyte®), which is licensed for meningeal relapse in NHL, has proved effective in treating lymphomatous and leukemic meningitis (Glantz *et al.* J Clin Oncol 1999;17:3110; Sancho et al. Haematologica 2006;91:ECR02). Intrathecal (IT) liposomal cytarabine is distributed throughout the CSF and has an extended half-life, allowing administration once every 2-4 weeks (Chamberlain *et al.* Arch Neurol 1995;52:912). Given the technical difficulty of performing timely lumbar punctures, the need for frequent administration of conventional intrathecal therapy with short half-life, and problems of compliance, especially in adults, it is logical to consider the use of liposomal cytarabine as CNS preventive therapy for patients with leukaemia (Ching Hon-Pui Leukaemia&Lymphoma, September 2007; 48(9):1672-3). In February 2007 we have thought to test the efficacy and the safety of liposomal cytarabine in CNS prophylaxis to patients with aggressive NHL and LAL. We have thought to follow the schedule that consider to use DepoCyte®, after four days high dose Ara-C or MTX and we performed intratecal every 3 or four weeks to avoid neurological toxicity. The prophylactic DepoCyte® was given too frequently especially with the concomitant high-dose chemotherapy treatment, in Jabbour study. In a subsequent study by McCkune DepoCyte® were given further apart (every3weeks) (Ching-Hong-Pui Leukemia &Lymphoma, September 2007; 48(9):1849-51). The findings of these studies suggest that liposomal cytarabine should not be given prior to or during treatment with high-dose chemotherapy that penetrates the blood-brain barrier. In all likelihood, liposomal cytarabine can be given safely after high-dose systemic chemotherapy (Ching-Hong-Pui, Leukemia &Lymphoma, September 2007, 48(9):1849-51). *Methods.* From February 2007, patients > 70 years of age were enroled. Diagnoses were:PZ1: 1 stage IA, IPI 1, Karnosky 75, diffuse large B-cell lymphoma (DLBCL) extra nodal testicular, riceceved R-COMP21; PZ2 1 stage IVA, IPI 2, HBV positive, Karnosky75, diffuse large B-cell lymphoma (DLBCL) extra nodal Para nasal sinus involvement and plus bone marrow riceved four cicles of R-COMP21+ 2 R 375mg/m^2 50% riduced dosage for age; PZ 3 Karnosky 50 LAL riceved first-line treatment HOLZER ALL01/81(Holzer 1984-88) dosage reduced for toxicity. All patients received CNS prophylaxis with IT liposomal cytarabine 50 mg followed by systemic steroid injection. All patientns we have performed flow cytometry and cytospin analysis of CSF every LP. In NHL cases, IT therapy was given the day before systemic chemotherapy for a total of 4 administrations; in LAL, prophylaxis was given every 3 weeks during induction for a total of 4 doses. *Results.* Three (2 NHL and 1 LAL) patients achieved a complete response (CR), with response durations. A median follow-up of eight months all patients were alive; 2 (1 DLBCL, 1 LAL) were in continuous CR. Isolated relapse of leukemia/lymphoma in the CNS was not seen. Liposomal cytarabine was well tolerated; no drug-related side effects or haematological toxicities were recorded. Conclusions:. Liposomal cytarabine should be the drug of choice for CNS prophylaxis, particularly in elderly patients.

PUB02

CORRELATION BETWEEN LEUKOCYTOSIS AND THROMBOSIS IN PHILADELPHIA NEGATIVE CHRONIC MYELOPROLIFERATIVE DISEASES

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Introduction. Recent investigations suggest that leukocytosis may cause thrombosis in Philadelphia negative (Ph-neg) MyeloProliferative Diseases (MPD). We investigated the relationship between leukocytosis and the occurrence of arterial and venous thromboembolic events in Ph-neg MPD patients over a period of two years. *Material and methods.* Seventy-five patients [46 females and 29 males, median age at diagnosis: 54 years; 42 with Essential Thrombocytemia (ET), 25 with Polycythaemia Vera (PV) and 9 with Idiopathic Myelofibrosis (IM)] were evaluated during the period 2000-2005; all of them received at least 2-years of follow-up. Patients were treated with cytoreductive therapy, anagrelide or -IFN accordingly to age and type of MPD. Twenty-one patients had at least one episode of objectively confirmed thrombosis (arterial or venous) at the moment of diagnosis, or six months prior to diagnosis or during the follow-up. *Results.* A total of 28 vascular events were observed: 12 (42.8%) occurred in patients with PV (3 in the follow-up), 13 (46.4%) in patients with TE (3 in the follow up) and 2 (7.1%) in patients with IM(1 in the follow up). A leukocytes count above 8.5×10^9/L (median value) was statistically associated with an increased risk of thrombosis (p= 0.03). The multivariate analysis, evaluating the interaction between conventional risk factors for thrombosis and leukocytosis, showed that the increased leukocytes counts was the most important risk factor for thromboembolic events. The relation between standard risk factors and leukocytosis is reported in the Table 1. *Conclusions.* The presence of a median leukocytes count >8.5×10^{9}L confers a high risk of thromboembolic eventsin Ph-negative MPD. These data may suggest the choice of cytoreductive therapy, but this approach must be confirmed in properly designed clinical trials, specially in young patients.

Table 1. Interaction between standard risk factors for thrombosis and leukocytes count.

*Risk factors**	*Hazars ratio (95% CI)*
Low risk with low WBC, n° 1/11, (9%)	1 (Reference)
Low risk with high WBC**, n° 4/7, (57%)	6.3 (4.1-8.4)
High risk with low WBC, n° 6/28, (21%)	2.3 (1.2-3.4)
High risk with high WBC, n° 18/29, (62%)	6.8 (2.9-10.7)

*Patients > 60 years and/or previous thrombosis; **Leukocytes > 8.5x10^9/L (median).

PUB03

TANDEM AUTOLOGOUS-SCT WITH Z-BEAM FOLLOWED BY REDUCED INTENSITY ALLOGENEIC STEM CELL TRANSPLANTATION AND DONOR-LYMPHOCYTE INFUSION IN REFRACTORY B-DIFFUSE LARGE CELL LYMPHOMA: A CASE REPORT

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Refractory DLCL have a very poor prognosis. Radioimmunotherapy with 90Y-Zevalin® plus BEAM (Z-BEAM) before ASCT has higher antilymphoma activity than conventional BEAM. Newertheless percentage of failures in this setting is still high. Allogeneic SCT is characterized by higher antilymphoma activity than ASCT, thanks to the graft-vs-lymphoma (GVL) effect; however also after allogenic rescue percentage of failures in refractory lymphomas is very high. Some reports suggest that a tandem approach with a debulking High-dose therapy (HDT), followed by minitransplant could be useful. We report here a case of young man with a mediastinal mass of 20 cm diameter which was diagnosed B-DLCL, CD20$^+$. This patient, enrolled in a Multicenter Protocol, received 4 R-MegaCHOP, with peripheral blood stem cell (PBSC) harvest, achieving Partial Remission (PR), but a rapid expansion of mediastinal mass, required rescue chemotherapy with 2 R-ICE; a PET-CT scan performed two weeks after showed disease progression so we directly

proceed to ASCT with Z-BEAM schedule. The post-transplant outcome was characterized by a rapid engraftment and varicella-zoster reactivation at day + 52. PET-CT at day +90 showed a stable disease with persistent positivity of the mediastinal mass, therefore patient undergo allogeneic SCT from a matched related donor, with conditioning regimen including Thiohepa-Fludarabine-Melphalan. We observed rapid and complete engraftment, complicated by pneumonia with pleural effusion and by encephalitis with complete clinical resolution within 1 month. A new PET-CT restaging at day +110 after allogeneic transplant showed reduction of mediastinal mass with persistent pathological Standard Uptake Value (SUV). Chimerism evaluation showed a full donor pattern, and a next PET-CT at day +180 showed further reduction of the mediastinal mass with normalization of SUV; response has been consolidated by DLI infusions with three doses of 1×10^6 $CD3^+$/kg monthly. Patient did not develop GVHD and at the last follow-up, (day +300 post allogeneic SCT) he is alive, well and in CR. This experience suggest that for refractory $CD20^+$ DLCL, the sequence including a first step with the maximization of anti-lymphoma effect (Z-BEAM) and a second step combining intermediate intensity conditioning with the immunological effect of T-donor lymphocytes, can overcome the lymphoma refractoriness and can achieve long term survival also in this very poor prognosis setting.

PUB04

AN UNUSUAL COMBINATION OF FOLLICULAR LYMPHOMA AND THROMBOCYTHEMIA-LIKE MYELOPROLIFERATIVE DISORDER WITH LIKELY MENINGIOMA

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A 73 years-old woman was admitted to our day-hospital with a 4-month history of left cervical lymphoadenomegaly. Physical examination showed also enlargement of right axillary nodes. Hepatosplenomegaly and B symptoms were absent. Blood cells count revealed WBC 7.0×10^3/microlitre, Hct 35.2%, Hgb 11.8 grams/decilitre, RBC 4.2×10^6/decilitre, Plt 1021×10^3/microlitre. Histological examination of left cervical nodal biopsy found a Follicular Lymphoma grade 3a. Bone marrow biopsy documented a Thrombocythemia-Like Myeloproliferative Disorder and the absence of lymphomatous infiltrate. Total body CT imaging studies revealed massive enlargement of intrathoracic and retroperitoneal nodes. In addition, an extracerebral intracranial mass was detected in the left occipital region. Radiologic features were more characteristic of meningioma, but secondary involvement by lymphoma was not ruled out. Surprisingly, the patient refused both diagnostic biopsy and MNR study of intracranial mass. Explorative lumbar puncture turned out negative and JAK2 V617F mutation was absent. Therefore, we diagnosed the coexistence of Follicular Lymphoma with clinical stage IIIA, FLIPI 5, likely Meningioma and Essential Thrombocythemia-Like Myeloproliferative Disorder. The patient received treatment with 6 courses of a combination of Rituximab-CHOP and antiaggregant therapy with acetylsalicylic acid. After the completion of treatment, CT scan showed complete disappearance of nodal enlargement and reduction of 33% of intracranial mass. Moreover, good control of myeloproliferative disease was obtained. The coexistence of Essential Thrombocythemia (ET) and non-Hodgkin's Lymphoma (NHL) is a rare event. We think that in these patients treatment should be tailored according to clinical characteristics of NHL. It's strictly necessary to treat NHL, if a high-grade or an advanced symptomatic low-grade lymphoma is diagnosed. On the other hand, it could be correct to treat myeloproliferative disorder if the NHL has an indolent course. The coexistence of NHL, ET and Meningioma is a truly rare condition. However, CT imaging studies are unable to clarify the differential diagnosis between these two entities, because an intracranial low-grade NHL may mimic a Meningioma, as reported in the literature. For this reason, our patient has now agreed to receive further investigations with the aim to define the nature of intracranial mass.

PUB05

IMMUNO-CHEMOTHERAPY WITH RITUXIMAB-CHOP IN A HEMODIALYSIS PATIENT WITH A NEWLY DIAGNOSED FOLLICULAR IIIA GRADE B-CELL NON-HODGKIN'S LYMPHOMA

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Till today few works are available on the treatment of hematological malignancies in patients with end stage renal disease (ESRD) and in persistent dialysis treatment. Though the administration of chemotherapy in combination with monoclonal antibody anti CD-20 rituximab is well established treatment in patients with non-Hodgkin's lymphoma with normal renal function, modest information on the safety and efficacy of the same are available for patients in ESRD. Here we describe one of the only some cases reported in the literature of a patient on haemodialysis, affects by non-Hodgkin's lymphoma large B-cell diffuse (DLBCL), who was treated with chemotherapy and rituximab (R-CHOP protocol) for a follicular lymphoma IIIa. A man of 70 year's old, diabetic, in dialysis for secondary diabetic nephropathy and affected by sarcoidosis, recently for appearance of itching, sweating, weight loss, fever and then brown excavated skin lesions was submitted to a skin biopsy that concluded for vasculitis. Subsequently when he arrived to our observation the clinical examination schowed presence of inguinal lymph nodes in the right with max diameter of 7 cm and he was submitted to inguinal lymph node biopsy that showed a non-Hodgkin lymphoma, follicular, grade IIIa. The computed tomography evidenced only lymph nodes enlarged at the right inguinal site. Bone biopsy was negative. Clinical staging was II B. He started the chemotherapy with R-CHOP protocol with a full dose of rituximab (375 mg/m^2) and reduction dose of 25% of chemotherapy. The dialysis was performed 24 hours after the chemotherapy. As more adverse event occurred a febrile neutropenia successfully treated with G-CSF and antibiotic therapy and moderate anaemia managed with epoetina alpha 40000 UI weekly. After the first cycle the patient resulted in a partial remission and the almost total disappearance of the skin lesions. The program provides 3-4 cycles and then RT IF. The administration of R-CHOP can be regarded as a safe therapeutic strategy in patients with EBDS and non-Hodgkin lymphoma although for precaution should be a reduction of doses at least 25%. A study had demoted that serum rituximab levels obtained before and after each treatment, before and after dialysis following each treatment, as well as in the dialysate fluid. The serum levels of rituximab were comparable to levels in patients with normal renal function. Rituximab is not eliminated by hemodialysis.

PUB06

A CASE OF "LATE RESPONDER": COMPLETE CYTOGENETIC REMISSION ON A PH^+ CML FOLLOWING A VERY LONG TERM LOW DOSE TREATMENT WITH IMATINIB MESYLATE

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Imatinib mesylate (IM) has become the gold standard treatment of Philadelphia-positive (Ph-pos) chronic myeloid leukaemia (CML). It induces complete cytogenetic remission (CCR) in 75-90% of patients in chronic phase (CP) and up to 40% of these patients obtain reduction of BCR/ABL transcript. Most of CCR are complete and stable and have a beneficial effect on survival. We here report a case of CCR on a Ph^+CML following a very long term low dose treatment with IM. In 1999 a 60-year-old women was admitted to our hospital for thrombocytosis (platet count 877 x10(9)/liter). On basis on bone marrow biopsy and cytogenetic analysis diagnosis of CML Ph-pos was performed (46 XX 10/10 Ph-positive metaphases). She began first hydroxyurea and next interferon alfa without cytogenetic or haematologic remission. Starting from February 2002, IM 400 milligrams (mg) daily was administrated. Two months later, patient developed a grade 2 neutropenia and IM dose was reduced (200-300 mg daily). We have monitored and analyzed the cytogenetic and molecular response to IM in this patient every six month in blood marrow specimen. Although lack of CCR, therapy was not modified because it was well tolerated and haematologic values persisted in normal range. Resistance to IM was excluded. Cytogenetic analysis have demonstrated a CCR (46 XX 0/6 Ph- positive metaphases) at 54 months

after initiation of IM. Residual disease was present: 35% BCR/ABL fusion protein transcript levels (method REAL TIME PCR). Therapy with IM can induce CCR and reduction of BCR-ABL transcript levels, but currently in case of IM resistance other drugs were administrated (dasatinib). In our observation prolonged IM administration even at low dose have induced CCR and molecular response with decrease of transcript levels. In the light of our report in Ph-Pos CML chronic phase long term therapy with IM can induce delayed CCR without therapy modification.

PUB07

RARE PLEURAL AND PULMONARY INVOLVEMENT IN NEWLY DIAGNOSED CLL

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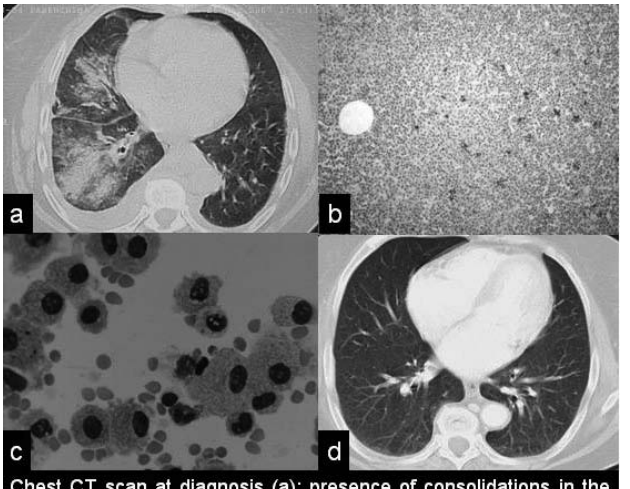
Pulmonary infiltration in chronic lymphocytic leukaemia (CLL) is rare. At autopsies parenchimal or pleural involvement is reported in about 40% of refractory/relapsed disease; instead the presence of clinical manifestations accompanied by radiological findings is extremely rare, expecially at diagnosis. A 65-year-old woman had respiratory distress with marked leukocytosis (291×10^3/microL), lymphocytosis (90%), mild anemia (Hb 11.1 g/dL), normal platelet count; she referred 10 kgs weight loss in three months and night sweating. Enlarged lymph nodes were palpable in the neck and the armpits, and hepatosplenomegaly was present. Arterial blood gas levels breathing room air were: pO2 62.7 mmHg, pCO2 30.3; pH 7.4, O2 sat. 93.2%. Pulmonary function tests showed: FVC 1.65 L, FEV1 1.51, FEV1/FVC 91 and TLC 5.15 L. CT scans showed right pleural effusion, thickening of the right diaphragmatic pleura and areas of consolidations in the middle and right lower lobes; moreover 2-cm-diameter lymphadenopathies in the armpits, supraclavicular regions, anterior mediastinum, retroperitoneum and inguinal stations were described and a homogeneous splenomegaly (90 cm^2).



Chest CT scan at diagnosis (a): presence of consolidations in the middle and right lower lobes, right pleural effusion. Bone marrow trephine biopsy (b): mature lymphoid infiltration, CLL. BAL (c): mature lymphocytes. Chest CT scan after Flu-Cy chemotherapy (d): complete remission of pleural and pulmonary findings.

Figure 1.

The patient underwent thoracentesis and bronchoscopy; bronchoalveolar lavage and pleural effusion fluids resulted negative for infections. The cytologic and cytofluorimetric examination, instead, revealed mature CD20$^+$, CD5$^+$, CD19$^+$, CD23$^+$ lymphocytes. Even PB smear showed mature lymphocytes and the imunophenotype 95% lymphocytes resulting positive for CD19, CD5, CD20, CD23, HLA-DR and negative for FMC, CD38 and sIg. Bone marrow (BM) aspiration consisted of 80% mature lymphoid elements and the trephine biopsy confirmed CD79A$^+$, CD5$^+$, CD23$^+$, ZAP70$^+$ CLL. Molecular biology revealed mutation of IgVH, the karyotype and FISH were normal. So CLL was diagnosed, stage C-Binet and III-Rai. FC chemotherapy was started (fludarabine 30 mg/m^2 and cyclophosphamide 300 mg/m^2 days 1-3). After 6 courses CT scan revealed the complete remission of all lymphoadenopaties and even of the pleural and pulmonary findings; the BM biopsy didn't show excess of lymphoid infiltration. This is a rare case of CLL presenting with lung and pleural infiltrations, which have been documented with non-invasive methods. After first line FC therapy a complete response was obtained, unlike the few other cases described, mainly regarding relapsing CLL and that had an unfavourable clinical outcome with different salvage therapies.

PUB08

EFFICACY OF BENDAMUSTINE MONOCHEMOTHERAPY IN AN OLD PATIENT AFFECTED BY A MULTIRESISTANT CLL COMPLICATED BY INFECTIVE AND HAEMOLITIC EPISODES

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In 2004 a 67 years old man came to our attention because of leukocytosis. The additional performed exams allowed to diagnostic CLL, stage 0 according Rai classification. Patients started a watch and wait program, however after 9 months lymphocytic population was dramatically increased and lymphonodal lesions were evident. Chlorambucil oral monochemotherapy, 10 mg/die for 10 consecutive days every month, was started. After 3 cycles, the patient experienced an additional slight increment of leukocytosis, positivity for Coombs' tests, with concomitant aptoglobulin reduction. The haemolitic complication did not allowed to use fludarabine as second line therapy, but a cyclophosphamide (CTX) and rituximab based regimen. Before the beginning of second line therapy the patient developed a lobar pneumonia and skin infection requiring hospitalization. After 2 months, at the resolution of pneumonia, CTX (iv, for 3 consecutive days every 21) and rituximab (every 21 days) chemotherapy was finally started. Second line therapy was complicated by the onset of penfigoid reaction that needed therapy suspension and steroid use, after 4 of the 6 planned cycles. The therapy allowed the resolution of peripheral lymphocytosis, but not of nodal lesions. At the time of cutaneous complication resolution, the patient experienced a new disease progression, with additional increment of nodal lesion. Despite previous infective complication, alemtuzumab therapy was started, with antinfective prophilaxis, 10 mg sc three times a week, till a cumulative dose of 240 mg. The patient did not achieve a significant response with residual 80% bone marrow infiltration, residual nodal lesions and mild leukocytosis. As forth line therapy, patient started CVP-rituximab regimen for 3 cycles when finally bendamustine was available at our center. Patient started bendamustine monochemotherapy, 100 mg/sq for 2 consecutive day, every 28 days. After 6 bendamustine cycles the patient obtained a complete response, with normal lymphocyte count, disappearance of nodal involvement as well as bone marrow infiltration. To consolidate the result the patient underwent 2 additional bendamustine cycles. Bendamustine chemotherapy was well tolerated, did not needed supportive therapies, did not induced infective episodes, was not compromised by previous use of alkilant agents such CTX, and most importantly allowed to reach for the first time after 4 therapeutic strategies, a complete response.

PUB09

PROPHYLACTIC AND THERAPEUTIC USE OF LIPOSOMAL CYTARABINE: A 7 CASES EXPERIENCE IN SINGLE INSTITUTION

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Central nervous system (CNS) involvement in patients with acute leukemia or aggressive non-Hodgkin lymphoma is a well known and dreadful complication. Standard treatment is based on the use of intratecal methotrexate, cytarabine and prednisone which have a short half-life in cerebrospinal fluid (CSF). Liposomal cytarabine is a prolonged release formulation providing cytotoxic concentrations for more than 14 days. From October 2006 to April 2008 we administered liposomal cytarabine to 7 patients (4 females and 3 males) followed in our Institution. The underlined diagnoses were acute lymphoblastic leukemia=3, M2 acute myeloid leukemia=1, high risk non-Hodgkin lymphoma=2 and lym-

phoid blastic crisis of chronic myeloid leukemia=1. Liposomal cytarabine was given as a 50 milligrams intratecal injection every two weeks with concomitant iv dexamethasone therapy in 3 very high risk patients as a prophylactic agent and in other 4 patients showing massive CNS presence of malignant cells in CSF as a therapeutic agent. The 3 patients receiving prophylactic injection were administered with 1, 2 and 5 doses respectively. The other 4 patients who underwent intratecal therapy were administered with 2, 3, 5 and 6 doses respectively. Seven out of 8 patients were receiving standard chemotherapy for their haematologic diseases. Intratecal liposomal cytarabine was well tolerated in all patients except for one who had to withdraw treatment after 5 doses due to the onset of headache, dizziness, severe lumbosciatalgy, muscle weakness and pains. The 3 patients with high risk leukemia-lymphoma who received liposomal cytarabin as prophylaxis did not show neoplastic meningitis during a median follow up of 80 days (range 64-99). About the 4 patients who received liposomal cytarabin as therapy two presented a progressive and complete clearance of malignant cells from CSF after two doses, one patient had no response and one case was not evaluable due to early death. The response was maintained for 6 and 97 days respectively until meningeal relapse occurred. Liposomal Ara-C proves to be a therapeutic option for patients with meningeal leukemia involvement. The use as CNS involvement prophylaxis in high risk patients seems to be rationale and should be evaluated in a controlled prospective trial.

PUB10

TREATMENT OF CN RELAPSE IN PH$^+$ ACUTE LYMPHOID LEUKEMIA PATIENT WITH LIPOSOMAL CYTARABINE. A CASE REPORT

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Despite the improvement in ALL treatment, the control of central nervous system (CNS) leukemia remains a therapeutic challenge. With current approaches, approximately 2% to 10% of patients can be expected to develop CNS relapse. Presenting features associated with an increased risk of CNS relapse include a T-cell immunophenotype, hyperleukocytosis, high-risk genetic abnormalities such as the Philadelphia chromosome and t(4;11), and the presence of leukemic cells in cerebrospinal fluid (even from iatrogenic introduction due to a traumatic lumbar puncture). Philadelphia chromosome-positive (Ph$^+$) acute lymphoblastic leukemia (ALL) is a high-risk, aggressive form of acute leukemia, with extremely poor prognosis, affecting primarily adults and the elderly. Liposomal cytarabine (DepoCyte®), a novel formulation with a terminal half-life 40 times longer than free cytarabine, has shown to be more effective than free cytarabine in patients with Leukemia/lymphomatous meningitis. We report here a case of a 60-year-old male patient with a ph$^+$ common B-ALL, diagnosed in September 2006. Cytogenetic analysis showed the translocation t(9;22)(q34;q11) in all metaphases and PCR analisys confirmed the BCR/ABL rearrangement. After induction chemotherapy + imatinib the patient obtained complete hematologic remission and then underwent to RIC allogeneic stem cell transplant. While bone marrow was still in complete hematological, cytogenetic and molecular remission the patient relapsed with leptomenigeal and parenchymal CNS disease. In december 2007 the patient had exophthalmos, and symptoms like dizziness, frontal headache and leg weakness. MRI showed two enhancing masses in optic nerve and in frontal lobe. A lumbar puncture confirmed the relapse (CD19/CD10 positive cells). PCR analisys performed in CSF confirmed the P190 BCR/ABL rearrangement. The salvage therapy with HD-MTX (3.5 g/mq/1day) +ARA-C (2 g/mq/bid/2days) and intratecal (IT) Depo Cyte® (50 mg every two weeks with concomitant dexametasone) was initiated and a rapid resolution of neurological symptoms occurred. We performed four IT injections every two weeks followed by further six injections every month. The CSF sample obtained before the second IT treatment showed no blast either with cytologic or cytofluorimetric analysis. PCR analisys confirmed the absence of P190 BCR/ABL rearrangement either in CSF or in bone marrow. No serious advers event do to DepoCyte® administration was observed. A RMN scan after 4 HD-MTX/ARA-C courses showed a very good partial response of optic nerve and frontal lobe masses and the patient was sheduled to underwent radiotherapy. Our experience confirmed the efficacy and of DepoCyte® in treatment of Leukemia meningitis, in association with systemic therapy, also in terms of compliance thanks to the DepoCyte® shedule of administration.

PUB11

AL AMYLOIDOSIS ASSOCIATED WITH CHRONIC LYMPHOCYTIC LEUKEMIA: A CASE REPORT

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AL Amyloidosis (AL-A) is caused by a clonal plasma cell dyscrasia, with progressive deposition of amyloid fibrils derived from monoclonal Ig light chains and multisystem organ failure. Rarely, AL-A can be associated with a B-cell lymphoproliferative disorder: Waldenstrom's macroglobulinemia, chronic lymphocytic leukaemia, or lymphoplasmacytic lymphoma. A 79-year-old man was admitted to our hospital in October 2000 because of urine proteins λ light chain restriction: smouldering myeloma was diagnosed by bone marrow biopsy without bone lytic lesions. In January 2007 the patient presented with weight loss, fatigue, peripheral edema, mild dispnea and hepatosplenomegaly. Laboratory examinations revealed: white blood cell 18200 leukocytes per millimeter cubic (lymphocytes 60 per cent, platelets count 69500 per millimeter cubic; hemoglobin 10 grams per decilitre); serum creatinine 1.26 milligrams per deciliter, Erythrocyte sedimentation rate was 50 millimeter first hour, beta 2 microglobulin 4,55 milligrams per decilitre. Serum immunoelectrophoresis showed IgG kappa paraproteinemia. Monoconal free light chains of serum were 23.4 milligrams per liter (kappa) and 247 milligrams per liter (lambda). NT-ProBNP 8039 nannograms per liter. At urine analysis: Proteinuria 9.02 grams per liter, with lambda light chain. Echocardiogram showed left ventricular ipertrophy with ejection fraction 40 percent. No lytic bone lesions on radiographic examinations. Bone marrow biopsy showed plasma cells 10-15 percent. Fat biopsy was positive with Congo Red and demonstrated Apple-Green Birefringence under polarized light. The diagnosis was cardiac and renal AL-A associated with B-CLL (classic immunophenotype). Combination melphalan predinosolone therapy was started. During treatment the patient developed infection of lower extremities with fever and congestive heart failure. After four months therapy minimal response was observed and thalidomide was started. Fourtheen months after diagnosis of cardiac and renal AL amyloidosis and B-LLC the patient is alive. *Conclusion*. AL-A associated with B-CLL is uncommon. These patients are on average older more likely to be female and present more commonly with multisystem amyloid disease than patients with disease associated with a plasma cell discrasia. Treatment decision are based primarly on the dominant hematopathologic features of the associated lymphoproliferative disorder.

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Editorial note

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Haematologica 2007;92:supplement no. 2 (XIth International Myeloma Workshop IVth International Workshop on Waldenström's Macroglobulinemia)

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Haematologica 2007;92:supplement no. 5 (7th International Symposium on Hodgkin Lymphoma, Cologne, Germany, 3–7 November 2007)

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2. Abstract Book: XIth International Myeloma Workshop & IVth International Workshop on Waldenstrom's Macroglobulinemia - 25-30 June 2007 - Kos Island, Greece. HAEMATOLOGICA-THE HEMATOLOGY JOURNAL 2007: 92 (Suppl 2). Published: JUN ~2007.

3. Abstract Book: 41st Congress of the Italian Society of Hematology - Bologna, Italy, October 14-17,2007. HAEMATOLOGICA-THE HEMATOLOGY JOURNAL 2007: 92 (Suppl 3). Published: OCT ~2007.

4. Abstract Book: XXXIV National Congress of the Italian Association of Pediatric Hematology Oncology. HAEMATOLOGICA-THE HEMATOLOGY JOURNAL 2007: 92 (Suppl 4). Published: OCT ~2007.

5. Abstract Book: 7th International Symposium on Hodgkin Lymphoma - 3-7 November 2007 - Cologne, Germany. HAEMATOLOGICA-THE HEMATOLOGY JOURNAL 2007: 92 (Suppl 5). Published: NOV ~2007.

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