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IX Congress of the Italian Society of Experimental Hematology

Napoli, Italy, September 20-22, 2006

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ABSTRACT BOOK

IX Congress of the Italian Society of Experimental Hematology

Napoli, Italy, September 20-22, 2006

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MAIN PROGRAM

PAROXYSMAL NOCTURNAL HEMOGLOBINURIA: A COMPLEX PATHOGENESIS DISEASE MODEL

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The recently discovered mutation of the *PIG-A* gene[1] has enabled the correct interpretation of most symptoms of the rare disease Paroxysmal Nocturnal Hemoglobinuria (PNH). Indeed, an impaired synthesis of the glicosyl phosphatidyl inositol (GPI) anchor causes the absence on PNH cell surface of as many as 30 GPI-linked molecules,[2] which in turn generates severe functional impairment in the various types of cell lineages involved. The most striking abnormality is an extreme hypersensitivity of red cells to complement activation, producing chronic intravascular hemolysis with paroxystic exacerbations, due to the absence of two complement inhibitor membrane molecules, CD59 and CD55. However, even PNH granulocytes and platelets have abnormal functions, leading to thrombosis. The discovery of so many membrane abnormalities has generated the possibility of using flow cytometry for diagnostic purposes, thus rendering the Ham test obsolete.

PNH has a number of intriguing peculiarities, and is therefore a model for understanding genetic, biochemical, immunological and therapeutic aspects that can somehow be applied to other less rare disorders. The following are relevant issues.

1. In PNH cells, the *PIG-A* gene has undergone a somatic mutation. Since this gene is on the X chromosome, a single mutational event is sufficient to determine the PNH phenotype in cells of both males (who are hemizygous) and females (who have a single active X chromosome). The molecular lesion is always severe (nonsense mutations, frameshift, deletion); [3] in the rare cases of missense mutations, the amino acids involved are located in critical molecular domains.

2. Mouse models have shown that knocking out the *pig-a* gene in the germinal line is incompatible with life,[4] chimerical mice, obtained through the use of *pig-a* knocked out embryonic stem cells, tend to lose the PNH cells soon after birth.[5] Thus, *pig-a* mutations do not confer any growth advantage.

3. Analyzing several million cells, there is evidence that even normal individuals may have a few PNH red cells and granulocytes in their circulating blood, but they do not develop PNH.[6] This confirms that a *PIG-A* mutation by itself is insufficient to cause the disease.

4. *PIG-A* mutations are *private* (i.e., each patient has his/her own type of mutation), and several PNH patients harbour more than a single *PIG-A* mutation (they may have two to four PNH clones.[7] Thus, the disease is clonal, but may not be monoclonal. The presence of multiple clones having the same gene altered in different molecular sites suggests clonal selection.

5. The inability of a cell to produce a small molecule needed to bridge bigger molecules on the outer membrane surface (or even on cytoplasmic organelles) generates a variety of cell changes, that involve several different cell functions. It has taken years to settle the clonal hypothesis with the variety of molecules lacking on PNH cell surface.

6. While PNH red cell half life is constantly shortened, PNH white cells and platelets have a normal lifetime. Nevertheless, PNH patients are often leucopenic and thrombocytopenic. Bone marrow is often hypoplastic, and in vitro growth of hematopoietic progenitors (BFU-E, CFU-G and GM, LTC-IC) is poor.[8,9] Attempts to mobilize CD34+ cells in PNH patients have failed.[10] Thus, all PNH patients have findings of an aplastic/hypoplastic disorder.

7. A way for bringing together these confounding findings is the dual hypothesis: every PNH patient must have a severe hypoplastic disorder and one or more PIG-A mutated stem cells.[11-13] What links these two issues is that PNH cells may be insensitive to the mechanism damaging the normal stem cells. Thus, a selection in favour of the PNH progenitors occurs, explaining both reduction of the normal stem cell pool and expansion of the PNH clone(s). This hypothesis also explains why the PIG-A lesion must be severe: only cells markedly deprived of GPI-linked molecules are supposed to be resistant to the mechanism damaging normal stem cells. By analogy with the most common aplastic/hypoplastic disorders, the killing mechanism is likely to be an immunological derangement involving T and/or NK cells. Indeed, recent evidence shows that autoimmune phenomena actively operate in PNH patients,[14-16] and that PNH cells are less susceptible to immunological damage.[17]

8. In order to explain PNH clonal expansion, there is no reason to postulate an underlying genetic instability at the *PIG-A* locus, or the occurrence of genetic lesions additional to the *PIG-A* mutation. The *private PIG-A* mutations likely occur at a rate not different from that of any of the somatic mutations naturally occurring in our genome,[18] but then there is a strong selective pressure favouring the mutated cell, which is operated by the mechanism impairing normal hematopoiesis.

9. An indirect confirmation of the *escape* theory comes from two clinical observations. First, a significant proportion of aplastic anemia (AA) patients may harbour a small PNH clone, even in the absence of clinically detectable hemolysis.[19] Second, the appearance of GPI-deprived lymphocytes has been described in patients treated by Campath, a monoclonal antibody targeting CD52, which is a GPI-linked molecule. During treatment, normal CD52+ lymphocytes are killed, while a few pre-existent PNH-like lymphocytes are protected by the absence of CD52, proliferate and expand in a clonal fashion. At treatment withdrawal, the selective pressure is removed and the GPI-deficient lymphocytes progressively disappear.[20] The pre-existence of PNH-like lymphocytes has been documented by molecular analysis.[21]

10. While for hyperhemolysis and marrow failure convincing pathogenic mechanisms have been demonstrated or proposed, there is still no definite biological explanation for thrombophilia, which mostly accounts for PNH patient mortality. Microparticles coming from activated PNH platelets or from intravascularly lysed red cells may be involved, as well as perturbation of the plasminogen/plasmin system due to uPAR, which is absent on PNH cells and increased in the plasma of PNH patients.

Several conclusions can be drawn from the above issues. First of all, the PNH population is not aggressive towards the organism; indeed, PNH is one of the few clonal non neoplastic disorders. Thus, target of possible treatments should not be the PNH clone(s), but rather the mechanism causing normal stem cell damage. Indeed, bone marrow transplantation and immunosuppressive therapy are the only curative treatments for this disorder. Approaches invoking gene therapy are not only difficult, but also nonsense, according to the *escape* theory: inducing the PNH cells to synthesize the GPI anchor means exposing them to the operating killing mechanism. Nonetheless, PNH cells are responsible for several symptoms of the disease, mainly those related to intravascular hemolysis, which may greatly affect patients' quality of life. How can we protect PNH red cells from complement activation? If we cannot modify red cell membrane abnormalities, can we fight against complement activation? Eculizumab (Soliris®, Alexion) is a new monoclonal antibody targeting the C5 fraction of the complement. In the absence of C5 cleavage, the complement terminal membrane attack complex C5-C9, which is responsible for hemolysis, cannot be formed. A pilot study,[22] a randomized trial[23] and two additional international studies have documented the clinical efficacy of this drug, the first specific treatment for PNH patients.

References

1. Takeda J, Miyata T, Kawagoe K, Iida Y, Endo Y, Fujita T, et al. Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. Cell 1993; 73: 703-11.
2. Rotoli B, Nafa K, Risitano AM. Paroxysmal nocturnal hemoglobinuria. In: Runge MD and Patterson C, ed. Principles of Molecular Medicine, 2nd edition. Philadelphia, Humana Press; 2006: 838-47.
3. Nafa K, Mason PJ, Hillmen P, Luzzatto L, Bessler M. Mutations in the PIG-A gene causing paroxysmal nocturnal hemoglobinuria are mainly of

the frameshift type. Blood 1995; 86: 4650-5.
4. Keller P, Tremml G, Rosti V, Bessler M. X inactivation and somatic cell selection rescue female mice carrying a Piga-null mutation. Proc Natl Acad Sci USA 1999; 96: 7479-83.
5. Tremml G, Dominguez C, Rosti V, Zhang Z, Pandolfi PP, Keller P, Bessler M. Increased sensitivity to complement and a decreased red blood cell life span in mice mosaic for a nonfunctional Piga gene. Blood 1999; 94: 2945-54.
6. Araten DJ, Nafa K, Pakdeesuwan K, Luzzatto L. Clonal populations of hematopoietic cells with paroxysmal nocturnal hemoglobinuria genotype and phenotype are present in normal individuals. Proc Natl Acad Sci USA 1999; 96: 5209-14.
7. Nishimura J, Inoue N, Wada H, Ueda E, Pramoonjago P, Hirota T, et al. A patient with paroxysmal nocturnal hemoglobinuria bearing four independent PIG-A mutant clones. Blood 1997; 89: 3470-6.
8. Rotoli B, Robledo R, Luzzatto L. Decreased number of circulating BFU-Es in paroxysmal nocturnal hemoglobinuria. Blood 1982;60 : 157-9.
9. Maciejewski JP, Sloand EM, Sato T, Anderson S, Young NS. Impaired hematopoiesis in paroxysmal nocturnal hemoglobinuria/aplastic anemia is not associated with a selective proliferative defect in the glycosylphosphatidylinositol-anchored protein-deficient clone. Blood 1997; 89: 1173-81.
10. Johnson RJ, Rawstron AC, Richards S, Morgan GJ, Norfolk DR, Hillmen SO. Circulating primitive stem cells in paroxysmal nocturnal hemoglobinuria (PNH) are predominantly normal in phenotype but granulocyte colony-stimulating factor treatment mobilizes mainly PNH stem cells. Blood 1998; 91: 4504-8.
11. Rotoli B, Luzzatto L. Paroxysmal nocturnal haemoglobinuria. Baillieres Clin Haematol 1989; 2: 113-38.
12. Dunn DE, Liu JM, Young NS. Paroxysmal nocturnal hemoglobinuria. In: Young NS, ed. Bone marrow failure syndromes. Philadelphia, W.B. Saunders; 2000: 99-121.
13. Luzzatto L, Bessler M, Rotoli B. Somatic mutations in paroxysmal nocturnal hemoglobinuria: a blessing in disguise? Cell 1997;88:1-4.
14. Risitano AM, Maciejewski JP, Green S, Plasilova M, Zeng W, Young NS. In vivo dominant immune responses in aplastic anemia patients: molecular tracking of putatively pathogenic T cells by TCR,-CDR3 sequencing. Lancet 2004; 364: 355-63.
15. Poggi A, Negrini S, Zocchi MR, Massaro AM, Garbarino L, Lastraioli S, et al. Patients with paroxysmal nocturnal hemoglobinuria have a high frequency of peripheral-blood T cells expressing activating isoforms of inhibiting superfamily receptors. Blood 2005; 106: 2399-408.
16. Chen G, Zeng W, Maciejewski JP, Kcyvanfar K, Billings EM, Young NS. Differential gene expression in hematopoietic progenitors from paroxysmal nocturnal hemoglobinuria patients reveals an apoptosis/immune response in *normal* phenotype cells. Leukemia 2005; 19: 862-8.
17. Murakami Y, Kosaka H, Maeda Y, Nishimura J, Inoue N, Ohishi K, et al. Inefficient response of T lymphocytes to glycosylphosphatidylinositol anchor-negative cells: implications for paroxysmal nocturnal hemoglobinuria. Blood 2002; 100: 4116-22.
18. Araten D, Luzzatto L. The mutation rate in PIG-A is normal in patients with paroxysmal nocturnal hemoglobinuria (PNH). Blood 2006; 108: 734-6.
19. Hillmen P, Lewis SM, Bessler M, Luzzatto L, Dacie JV. Natural history of paroxysmal nocturnal hemoglobinuria. N Engl J Med 1995;333:1253-8.
20. Hertenstein B, Wagner B, Bunjes D, Duncker C, Raghavachar A, Arnold R, et al. Emergence of CD52-, phosphatidylinositolglycan-anchor-deficient T lymphocytes after in vivo application of Campath-1H for refractory B-cell non-Hodgkin lymphoma. Blood 1995; 86: 1487-492.
21. Rawstron AC, Rollinson SJ, Richards S, Short MA, English A, Morgan GJ, et al. The PNH phenotype cells that emerge in most patients after CAMPATH-1H therapy are present prior to treatment. Br J Haematol 1999; 107: 148-53.
22. Hillmen P, Hall C, Marsh JC, Elebute M, Bombara MP, Petro BE, et al. Effect of eculizumab on hemolysis and transfusion requirements in patients with paroxysmal nocturnal hemoglobinuria. N Engl J Med 2004; 350: 552-9.
23. Hillmen P, Young NS, Schubert J, Brodsky RA, Socié G, Muus P, et al. Safety and efficacy of the terminal C5 complement inhibitor eculizumab in patients with paroxysmal nocturnal hemoglobinuria. N Engl J Med, 2006, in press.

MICRORNAS IN THE REGULATION OF NORMAL AND NEOPLASTIC HEMATOPOIESIS

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Hematopoiesis is highly controlled by lineage-specific transcription factors that by interacting with specific DNA sequences directly activate or repress specific gene expression. Lineage-specific transcription factors have been found mutated or altered by chromosomal translocations associated to leukemias, indicating their role in the pathogenesis of these malignancies.[1-4] The post genomic era however, has shown that transcription factors are not the unique key regulators of gene expression. Epigenetic mechanisms such as DNA methylation, post-translational modifications of histones, remodeling of nucleosomes and expression of small regulatory RNAs all contribute to the regulation of gene expression and determination of cell and tissue specificity[5,6] Deregulation of these epigenetic mechanisms, indeed cooperate with genetic alterations to the establishment and progression of tumors.

MicroRNAs (miRNAs) are a new class of small noncoding RNAs that bind to their targeted mRNAs. According to the degree of mismatch between the miRNA and the 3'UTR region on mRNAs, miRNAs either block mRNA translation or induce mRNA degradation.[7] miRNAs act as negative regulators of the expression of genes involved in development, differentiation, proliferation and apoptosis. Their expression is tissue-specific and highly regulated according to the cell's developmental stage.[7] miRNAs are differentially expressed in hematopoietic cells *in vivo*,[8] thus suggesting their role in hematopoiesis and lineage differentiation. Several experimental evidences support this hypothesis, including: i) ectopic expression of miR-181 in mouse hematopoietic/stem progenitors cells increases the fraction of B-lineage cells *in vitro* and *in vivo*[8]; ii) miRNAs expressed in hematopoietic cells (ie miR-15, miR-16) have been found mutated or altered by chromosomal translocations or deletions associated to leukemias[9]; iii) down-modulation of miR-221 and miR-222 expression levels promotes erythropoiesis of human $CD34^+$ progenitor cells[10]; iv) up-regulation of miR-223 in human myeloid progenitors promotes granulocytic differentiation[11]; Moreover, wide screening approaches performed to establish miRNA expression profiles show a unique miRNA signature relevant for the pathogenesis, diagnosis and prognosis of myeloid and lymphoid leukemias.[9,12]

The activation of both pathways of transcriptional regulation by the myeloid lineage-specific transcription factor CCAAT/enhancer binding protein α (C/EBPα), and post-transcriptional regulation by miR-223 appears essential for granulocytic differentiation and clinical response of APL blasts to ATRA. Mir-223 induction decreases the protein levels of Nuclear Factor IA (NFI-A), which is one of its target mRNAs. NFI-A is a member of the CCAAT-box binding transcription factor NFI family of proteins (also including NFI-B,-C and -X) and acts as a regulator of gene expression, implicated replication and in cell growth.[13] Interestingly, NFI-A is able to compete with the C/EBPα in binding the CCAAT element on promoters.[14] Consistent with these evidences, RA treatment recruits C/EBPα on the CCAAT elements present on miR-223 promoter and displaces NFI-A from its binding site allowing up-regulation of miR-223 expression and granulocytic differentiation of APL blasts.[11]

Together these evidence underlies transcription factors, chromatin remodeling and miRNAs as ultimate determinants for the correct organization of cell-type specific gene arrays and hematopoietic differentiation therefore providing new targets for the diagnosis and treatment of leukemias.

References

1. Rabbitts TH. Chromosomal translocations in human cancer. Nature 1994;372:143-9.
2. Tenen DG. Disruption of differentiation in human cancer: AML shows the way. Nat Rev Cancer 2003;3:89-101.
3. Salomoni P, Pandolfi PP. Transcriptional regulation of cellular transformation. Nat Med 2000;6:742-744.
4. Cantor AB, Orkin SH. Transcriptional regulation of erythropoiesis: an affair involving multiple partners. Oncogene 2002;21:3368-76.
5. Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci 2006;31:89-97.
6. Bernstein E, Allis CD. RNA meets chromatin. Genes Dev 2005;19:1635-55.
7. Bartell DP. MicroRNAs: genomics, biogenesis, mechanism and function.Cell. 2004;116:281-297.
8. Chen C-Z, Li L, Lodish HF, Bartell DP. MicroRNAs Modulate Hematopoietic Lineage Differentiation. Science 2004;303:83-86.
9. Calin GA, Ferracin M, Cimmino A et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 2005;353:1793-801.
10. Felli N, Fontana L, Pelosi E et al. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. Proc Natl Acad Sci USA 2005;102:18081-6.
11. Fazi F, Rosa A, Fatica A et al. A Minicircuitry Comprised of MicroRNA-223 and Transcription Factors NFI-A and C/EBPalpha Regulates Human Granulopoiesis. Cell 2005;123:819-31.
12. Lu J, Getz G, Miska EA et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834-8.

13. Gronostajski RM. Roles of the NF1/CTF gene family in transcription and development. Gene 2000;249:31-45.
14. Garlatti M, Tchesnokov V, Daheshia M et al. CCAAT/enhancer-binding protein-related proteins bind to the unusual promoter of the aspartate aminotransferase housekeeping gene. J Biol Chem 1993;268:6567-74.

GENE EXPRESSION SIGNATURE OF LEUKEMIC STEM CELLS IN MODELS OF ACUTE MYELOID LEUKEMIA

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The pathogenesis of acute myeloid leukemia (AML) is linked to oncogenic fusion proteins generated as a consequence of chromosomal translocations, found >50 % of AML cases.[1] Despite genetic heterogeneity, there is increasing evidence for common molecular and biological mechanisms underlying acute myeloid leukemogenesis.[2] AML fusion proteins function as aberrant transcriptional regulators that interfere with myeloid differentiation and determine a stage-specific arrest of maturation. The abnormal regulation of transcriptional networks occurs through similar mechanisms that include recruitment of aberrant co-repressor complexes, alterations in chromatin remodeling, and disruption of specific subnuclear compartments. Approximately 35% of AMLs are instead associated with point mutations of the nucleophosmin (NPM) gene, which determine cytoplasmic localization of the NPM protein (NPMc+ AML).[3] The mechanism of leukemogenesis triggered by NPM mutants is unknown, but is unlikely to depend on direct transcriptional regulation. The precise nature of the hematopoietic cell targeted by the leukemogenic event is not known, although several investigations indicate that transformation occurs in very early progenitors.[4] Leukemic stem cells (LSC), which are believed to represent a reservoir of leukemic cells with a low proliferative rate and ability to self-renew, may derive from transformed hematopoietic stem cells (HSC) or from more committed precursors that have re-acquired stem cell characteristics.[5]

We have started a series of investigations aimed at characterizing the molecular signature of LSC and identifying specific markers for disease initiation and progression. In order to gain insight into the molecular signature of LSCs, we have analyzed gene expression profiles in diverse types of AML models and identified a set of putative stem cell regulators that are abnormally expressed in leukemia. Our results reveal that specific AML fusion proteins (AML1/ETO, PML/RARα) are capable of directly activating genes involved in maintenance of the stem-cell phenotype, and repressing genes that regulate hematopoietic stem cell (HSC) commitment or differentiation, suggesting that fusion proteins may determine the activation/maintenance of stem cell circuits in LSC.

We have integrated gene expression studies to genome-wide chromatin immunoprecipitation assays with the aim of investigating the molecular basis of transcriptional regulation in AML. By merging the data sets, we have identified genes whose expression is deregulated by direct binding of fusion proteins to regulatory regions. Surprisingly, in contrast to what is expected from transcriptional repressors, a relevant proportion of genes are induced by AML fusion proteins, suggesting that AML fusion proteins can also act as transcriptional activators through yet unknown mechanisms. Specific chromatin "marks", such as acetylation or methylation of histone lysine residues, are associated to DNA regions that are recognized by oncogenic transcription factors.

NPMc+ leukemias display a specific gene expression profile dominated by a "stem cell" molecular signature, and in particular by the activation of numerous members of homeodomain-containing transcription factors, including HOX and TALE genes, some of which are implicated in hematopoietic development, and might reflect the molecular status of the LSC rather than represent a direct consequence of NPM mutations.

References

1. Look, A.T. (1997). Oncogenic transcription factors in the human acute leukemias. Science 278, 1059-1064.
2. Alcalay, M., Orleth, A., Sebastiani, C., Meani, N., Chiaradonna, F., Casciari, C., et al. (2001). Common themes in the pathogenesis of acute myeloid leukemia. Oncogene 20, 5680-5694.
3. Falini, B., Mecucci, C., Tiacci, E., Alcalay, M., Rosati, R., Pasqualucci, L., et al. (2005). Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med 352, 254-266.
4. Bonnet, D., and Dick, J.E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3, 730-737.
5. Passegue, E., Jamieson, C.H., Ailles, L.E., and Weissman, I.L. (2003). Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? Proc Natl Acad Sci U S A 100 Suppl 1, 11842-11849.

NEOANGIOGENESIS AND MICROVASCULATURE IN MULTIPLE MYELOMA: ITS ROLE IN LEUKEMIC PROGRESSION

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Angiogenesis is a constant hallmark of multiple myeloma (MM) progression and has prognostic potential. It is induced by plasma cells via angiogenic factors with the transition from monoclonal gammopathy of undetermined significance (MGUS) to MM, and probably with loss of angiostatic activity on the part of MGUS.[1] The pathophysiology of MM-induced angiogenesis is complex and involves both direct production of angiogenic cytokines by plasma cells and their induction within the microenvironment.[2] The cause of induction of the vascular phase is the subject of current investigation.[1] Several studies show overexpression and secretion of vascular endothelial growth factor (VEGF) by the clonal plasma cells.[3] VEGF stimulates proliferation and chemotaxis in both endothelial cells (ECs) via VEGF receptor-2 (VEGFR-2) and stromal cells (BMSCs) via VEGFR-1. These cells are rapidly phosphorylated by the interaction with VEGF, and signal via extracellular signal-related kinase-2 (ERK-2).[3] It is clear, therefore, that simultaneous high coexpression of VEGF and VEGFR-2, constitutive autophosphorylation in VEGFR-2 and ERK-2, and its blockade along with proliferation by anti-VEGF and even more by anti-VEGFR-2 neutralizing mAbs provide convincing evidence of the presence of an operative autocrine loop for VEGF in MM ECs.[4] The greater effectiveness of VEGFR-2 blockade may be due to its prevention of receptor activation by both extracellular and intracellular VEGF, whereas the anti-VEGF mAbs only targets the extracellular form. Cytokines are secreted by BMSCs, ECs and osteoclasts, and promote plasma cell growth, survival and migration, as well as paracrine cytokine secretion and angiogenesis in the bone marrow milieu.[2] Angiogenesis is also supported by inflammatory cells following their recruitment and activation by plasma cells. BMSCs may act synergistically with tumor cells by secreting the same or other angiogenic factors.[2] Mast cells play a pivotal role in this synergism, because they are a rich source of preformed and newly-synthesized angiogenic cytokines and growth factors, such as tumor necrosis factor alpha (TNF-α), interleukin-8 (IL-8), fibroblast growth factor-2 (FGF-2) and VEGF, and proteases, such as tryptase and chymase, which are contained in their secretory granules. In fact, bone marrow angiogenesis, evaluated as microvessel area, and mast cell counts are highly correlated in patients with nonactive and active MM and in those with MGUS, and both parameters increase simultaneously in active MM.[2] Recently, we have shown that the VEGF-driven angiogenic potential of MM ECs is significantly higher than that of MGUS ECs, and probably due to a constitutive imbalance of endogenous VEGF/semaphorin 3A (SEMA3A) ratio, which leans on VEGF in MM ECs but on SEMA3A in MGUS ECs.[5] Exogenous VEGF induces SEMA3A expression in MGUS ECs, but not in MM ECs. Moreover, by counteracting VEGF activity as efficiently as an anti-VEGFR-2 antibody, exogenous SEMA3A restrains the over-angiogenic potential of MM ECs.[5] Our data indicate that loss of endothelial SEMA3A in favor of VEGF could be responsible for the angiogenic switch from MGUS to MM. Finally, circulating ECs and endothelial precursor cells (EPCs) contribute to the neovascularization, and the presence of EPCs suggests that vasculogenesis (new vessel formation from EPCs) may also contribute to the full MM vascular tree and progression.[6]

References

1. Vacca A, Ribatti D, Bone marrow angiogenesis in multiple myeloma. Leukemia 2006; 20; 193-9.
2. Ribatti D, Nico B, Vacca A, Importance of the bone marrow microenvironment in inducing the angiogenic response in multiple myeloma. Oncogene 2006 (in press).
3. Ria R, Roccaro AM, Merchionne F, Vacca A, Dammacco F, Ribatti D, Vascular endothelial growth factor and its receptors in muliple myeloma. Leukemia 2003; 17: 1961-6.
4. Ria R, Vacca A, Russo F, et al., A VEGF-dependent autocrine loop mediates

proliferation and capillarogenesis in bone marrow endothelial cells of patients with multiple myeloma. Thromb Haemost 2004; 92: 1438-45.
5. Vacca A, Scavelli C, Serini G, et al. Loss of inhibitory semaphorin 3A (SEMA3A) autocrine loops in bone marrow endothelial cells of patients with multiple myeloma Blood 2006; (in press).
6. Ribatti D, Nico B, Crivellato E, Vacca A, Endothelial progenitor cells in health and disease. Histol Histopathol 2005; 20: 1351-8.

ANAPLASTIC LYMPHOMA KINASE: MECCANISMI MOLECOLARI E TRASFORMAZIONE LINFOIDE"

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Anaplastic large cell lymphomas (ALCL) represent a unique subset of lymphomas characterized by the CD30 expression and associated with specific chromosome translocations in which the Anaplastic Lymphoma Kinase (ALK) gene is fused to several partners, most frequently to the NPM gene. Activated ALK chimeras bind multiple adaptor proteins capable of firing several regulatory pathways controlling cell proliferation, survival, and transformation. We have previously demonstrated that the constitutive expression and phosphorylation of ALK chimeric proteins is sufficient for cellular transformation, and its activity is strictly required for the survival of ALCL cells, *in vitro* and *in vivo*. To clarify molecular mechanisms and signalling pathways required for NPM-ALK-mediated transformation and tumour maintenance, we analyzed the transcriptomes of ALK positive ALCL cell lines using experimentally controlled approaches, in which ALK signaling was abrogated by a doxycycline-inducible ALKshRNA or by specific ALK kinase inhibitors. Inducible NPM-ALK knockdown or inhibition of ALK enzymatic activity resolved in the modulation of multiple downstream effectors, followed by growth arrest, apoptosis, and tumor regression of xenograft ALCL tumors. The combined analysis of NPM-ALK modulated genes by microarray gene expression profiling identified known and novel downstream targets, grouped in functional clusters of cell cycle and proliferation, adhesion and migration, and in cytokine signalling family molecules. In a functional screen of NPM-ALK regulated genes, we found that the anti-apoptotic protein Bcl2A1 and the transcription factor C/EBPb are strictly regulated by NPM-ALK activity and they sustain the survival and/or growth of ALK positive ALCL cells. Overall, the combination of an experimentally controlled gene expression profiling analysis with a functional RNA interference screening represents a powerful tool to characterize the network mediating tumorigenesis and maintenance of lymphomas. Moreover, this approach will open new avenues to identify suitable targets for specific therapeutic strategies.

NOVEL TYROSINE KINASE INHIBITORS FOR PH+ LEUKEMIA PATIENTS RESISTANT TO IMATINIB

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Despite the excellent clinical results obtained with the Bcr-Abl tyrosine kinase inhibitor imatinib mesylate, there is still a need to improve therapy for patients with chronic myeloid leukemia (CML) and Philadelphia-positive (Ph+) acute lymphoblastic leukemia (ALL). Imatinib induces complete hematologic and cytogenetic remissions in most newly diagnosed CML patients,[1] but relatively few of them achieve molecular remission. In addition, imatinib is much less effective in advanced phase-CML as well as in Ph+ ALL, mainly due to the development of drug resistance.[2] Resistance to imatinib is most commonly mediated by the emergence of Abl kinase domain mutations which target critical contact points between imatinib and Bcr-Abl or induce a transition from the inactive to the active conformation of the kinase, to whom imatinib is unable to bind. The clinical impact of mutations varies according to their different degree of residual sensitivity to imatinib. Indeed, while certain Bcr-Abl mutations retain *in vitro* sensitivity to imatinib at physiologically relevant concentrations and therefore may not be clinically meaningful, other require increased doses of imatinib, and some confer a highly resistant phenotype (Table 1).[3] Approximately 10% of patients with CML in chronic phase (CP) treated with imatinib front-line, 20% of CP-CML patients treated with imatinib after interferon failure, 45% of accelerated phase patients, 70% of myeloid blast crisis and 80% of lymphoid blast crisis and Ph+ ALL patients have evidence of point mutations within the ABL kinase domain at the time of resistance to imatinib.4 In the remaining patients, the reasons for imatinib resistance have to be traced to Bcr-Abl gene amplification or overexpression, clonal cytogenetic evolution, or altered levels of transport molecules responsible for imatinib influx and efflux (ABC transporters, hOCT1).[2] To counteract the problem of resistance due to point mutations, several second-generation inhibitors have been synthesized and tested in pre-clinical assays: nilotinib (AMN107)5-9; dasatinib (BMS-354825);6,10-14 SKI-606;15 VX-680;12,16 AP23464;17,18 NS-187;19,20 PD166326, PD180970 and PD173955;21-24 ON012380.25 The imatinib derivative nilotinib and the dual-specificity SRC/ABL inhibitor dasatinib are currently being evaluated in phase II clinical trials and are close to registration; phase I trials of SKI-606 have just started. Second-generation compounds have increased potency with respect to imatinib and are active against the vast majority of the clinically relevant Bcr-Abl mutant forms, but most of them have invariably been shown to be unable to overcome the T315I. Structural analyses indicate that the substitution of threonine with isoleucine at residue 315 (the so-called *gatekeeper residue*) eliminates a crucial hydrogen-bonding interaction and introduces a steric hindrance which abrogates binding and effective inhibition of Bcr-Abl by the inhibitors.[13,24] The T315I is responsible for approximately 15% of the cases of relapse in CML and Ph+ ALL patients on imatinib therapy,[4] but the clinical relevance and the incidence of this mutant is dramatically increasing since it is selected as the main mechanism of resistance to dasatinib and nilotinib.[9,14] Therefore, there is an urgent need for the development of compounds active against the T315I-Bcr-Abl mutant. A possible approach to the development of second-line strategies overcoming resistance induced by the T315I mutation is to design inhibitors binding regions of Bcr-Abl other than the ATP binding pocket. This is the case of ON012380, which binds to the substrate binding site and exhibited low nanomolar activity against the T315I mutant in biochemical and cellular assays.[25]

Table 1. Comparison between imatinib, dasatinib and nilotinib IC50 values obtained in Ba/F3 cellular proliferation assays. Adapted from 6.

	Cellular proliferation					
Abl variant	*imatinib*		*nilotinib*		*dasatinib*	
	IC50 (nM)	*fold-change*	*IC50 (nM)*	*fold-change*	*IC50 (nM)*	*fold-change*
Wild-type	260	1	13	1	0.8	1
M244V	2000	8	38	3	1.3	2
G250E	1,350	5	48	4	1.8	2
Q252H	1,325	5	70	5	3.4	4
Y253F	3,475	13	125	10	1.4	2
Y253H	>6400	>25	450	35	1.3	2
E255K	5200	20	200	15	5.6	7
E255V	>6400	>25	430	33	11	14
F311L	480	2	23	2	1.3	2
T315I	>6400	>25	>2000	>154	>200	>250
F317L	1,050	4	50	4	7.4	9
M351T	880	3	15	1.2	1.1	1.4
F359V	1,825	7	175	13	2.2	3
L387M	1,000	4	49	4	2	3
H396P	850	3	41	3	0.6	0.8
H396R	1,750	7	41	3	1.3	2

An intriguing alternative is to explore the possibility of whether molecules that have been developed as inhibitors for other protein kinases and are already undergoing clinical trials might include the T315I-Bcr-Abl mutant among their *off-targets*. Although *off-target* activity may lead to undesirable side effects, it has to be recognized that focusing on compounds that are already being tested in the clinic may expedite the development of successful therapeutic strategies. Recent studies have shown that MK-0457 (VX-680), a small-molecule originally developed as an aurora kinase inhibitor, has *in vitro* activity against the T315I-Bcr-Abl at micromolar concentrations.12,16 MK-0457 binds to residues involved in

ATP-binding and catalysis which are highly conserved across tyrosine and serine/threonine kinases, and co-crystal studies have documented that the inhibitor does not penetrate in the kinase domain so deeply as imatinib – a close encounter with the *gatekeeper* is therefore avoided, explaining the ability of the drug to accommodate the substitution of isoleucine for threonine at residue 315. The remarkable efficacy of MK-0457 raises the question of whether aurora kinases may also harbor some pathogenetic significance in CML and/or Ph+ ALL or may selectively be deregulated by the T315I-Bcr-Abl, and whether auroras may be a suitable secondary target for inhibition.

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References

1. Druker B, Guilhot F, O'Brien S G, Larson R. Long-term benefits of imatinib (IM) for patients newly diagnosed with chronic myelogenous leukemia in chronic phase (CML-CP): the 5-year update from the IRIS study. J Clin Oncol, 2006 ASCO Annual Meeting Proceedings 2006;24:6506.
2. Hochhaus A, Hughes T. Clinical resistance to imatinib: mechanisms and implications. Hematol Oncol Clin North Am 2004;18:641-66.
3. Corbin AS, La Rosee P, Stoffregen EP, Druker BJ, Deininger MW. Several Bcr-Abl kinase domain mutants associated with imatinib mesylate resistance remain sensitive to imatinib. Blood 2003;101:4611-4.
4. Soverini S, Colarossi S, Gnani A, Rosti G, Castagnetti F, Poerio A, et al. Frequency, distribution and prognostic value of ABL kinase domain (KD) mutations in different subsets of Philadelphia-positive (Ph+) patients (pts) resistant to Imatinib (IM) by the GIMEMA Working Party on CML. Blood 2005;106:435.
5. Golemovic M, Verstovsek S, Giles F, Cortes J, Manshouri T, Manley PW, et al. AMN107, a novel aminopyrimidine inhibitor of Bcr-Abl, has in vitro activity against imatinib-resistant chronic myeloid leukemia. Clin Cancer Res 2005;11:4941-7.
6. O'Hare T, Walters DK, Stoffregen EP, Jia T, Manley PW, Mestan J, et al. In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. Cancer Res 2005;65:4500-5.
7. Weisberg E, Manley PW, Breitenstein W, Bruggen J, Cowan-Jacob SW, Ray A, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell.ì 2005;7:129-41.
8. Weisberg E, Manley P, Mestan J, Cowan-Jacob S, Ray A, Griffin JD. AMN107 (nilotinib): a novel and selective inhibitor of BCR-ABL. Br J Cancer 2006.
9. Kantarjian H, Giles F, Wunderle L, Bhalla K, O'Brien S, Wassmann B, et al. Nilotinib in imatinib-resistant CML and Philadelphia chromosome-positive ALL. N Engl J Med 2006;354:2542-51.
10. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. Science 2004;305:399-401.
11. Burgess MR, Skaggs BJ, Shah NP, Lee FY, Sawyers CL. Comparative analysis of two clinically active BCR-ABL kinase inhibitors reveals the role of conformation-specific binding in resistance. Proc Natl Acad Sci USA 2005;102:3395-400.
12. Carter TA, Wodicka LM, Shah NP, Velasco AM, Fabian MA, Treiber DK, et al. Inhibition of drug-resistant mutants of ABL, KIT, and EGF receptor kinases. Proc Natl Acad Sci USA 2005;102:11011-6.
13. Tokarski JS, Newitt JA, Chang CY, Cheng JD, Wittekind M, Kiefer SE et al. The structure of Dasatinib (BMS-354825) bound to activated ABL kinase domain elucidates its inhibitory activity against imatinib-resistant ABL mutants. Cancer Res 2006;66:5790-7.
14. Talpaz M, Shah NP, Kantarjian H, Donato N, Nicoll J, Paquette R, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N Engl J Med 2006;354:2531-41.
15. Golas JM, Arndt K, Etienne C, Lucas J, Nardin D, Gibbons J, Frost P, Ye F, Boschelli DH, Boschelli F. SKI-606, a 4-anilino-3-quinolinecarbonitrile dual inhibitor of Src and Abl kinases, is a potent antiproliferative agent against chronic myelogenous leukemia cells in culture and causes regression of K562 xenografts in nude mice. Cancer Res. 2003;63:375-381
16. Young MA, Shah NP, Chao LH, Seeliger M, Milanov ZV, Biggs WH 3rd, et al. Structure of the kinase domain of an imatinib-resistant Abl mutant in complex with the Aurora kinase inhibitor VX-680. Cancer Res. 2006;66:1007-14.
17. O'Hare T, Pollock R, Stoffregen EP, Keats JA, Abdullah OM, Moseson EM, et al. Inhibition of wild-type and mutant Bcr-Abl by AP23464, a potent ATP-based oncogenic protein kinase inhibitor: implications for CML. Blood 2004;104:2532-9.
18. Azam M, Nardi V, Shakespeare WC, Metcalf CA 3rd, Bohacek RS, Wang Y, et al. Activity of dual SRC-ABL inhibitors highlights the role of BCR/ABL kinase dynamics in drug resistance. Proc Natl Acad Sci USA. 2006.
19. Kimura S, Naito H, Segawa H, Kuroda J, Yuasa T, Sato K, et al. NS-187, a potent and selective dual Bcr-Abl/Lyn tyrosine kinase inhibitor, is a novel agent for imatinib-resistant leukemia. Blood. 2005;106:3948-54.
20. Naito H, Kimura S, Nakaya Y, Naruoka H, Ito S, Wakayama T, et al. In vivo antiproliferative effect of NS-187, a dual Bcr-Abl/Lyn tyrosine kinase inhibitor, on leukemic cells harbouring Abl kinase domain mutations. Leuk Res 2006.
21. Dorsey JF, Jove R, Kraker AJ, Wu CJ. The pyrido[2,3-d] pyrimidine derivative PD180970 inhibits p210Bcr-Abl tyrosine kinase and induces apoptosis of K562 leukemic cells. Cancer Res 2000;60:3127-31.
22. Huron DR, Gorre ME, Kraker AJ, Sawyers CL, Rosen N, Moasser MM. A novel pyridopyrimidine inhibitor of abl kinase is a picomolar inhibitor of Bcr-abl-driven K562 cells and is effective against STI571-resistant Bcr-abl mutants. Clin Cancer Res 2003;9:1267-73.
23. La Rosee P, Corbin AS, Stoffregen EP, Deininger MW, Druker BJ. Activity of the Bcr-Abl kinase inhibitor PD180970 against clinically relevant Bcr-Abl isoforms that cause resistance to imatinib mesylate (Gleevec, STI571). Cancer Res 2002;62:7149-53.
24. Nagar B, Bornmann WG, Pellicena P, Schindler T, Veach DR, Miller WT, Clarkson B, Kuriyan J. Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). Cancer Res 2002;62:4236-43.
25. Gumireddy K, Baker SJ, Cosenza SC, John P, Kang AD, Robell KA, Reddy MV, Reddy EP. A non-ATP-competitive inhibitor of BCR-ABL overrides imatinib resistance. Proc Natl Acad Sci USA 2005;102:1992-7.

NEW INSIGHTS ON DNA METHYLATION AND GENE EXPRESSION IN HEMATOLOGICAL MALIGNANCIES

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Epigenetic modifications are potentially reversible DNA and chromatin modifications transmitted from a cell to its progeny, able to induce altered gene expression without changing DNA sequence and without any new genetic information. Such modifications involve chromatin organization by DNA methylation and histone acetylation, methylation and phosphorylation. Methylation of DNA is a biochemical modification that is responsible for embryogenesis, cell differentiation, imprinting, X chromosome inactivation and cancer. Evidence that has accumulated in the past years suggests that cancer cells usurp this physiologic mechanism and use it to their benefit by silencing tumour suppressor genes or genes responsible for cell maturation and apoptosis and related proteins, whose primary structure remains intact. DNA methylation is a covalent chemical modification, adding a CH3 group at the carbon 5 position of cytosine situated in the sequence contest 5'CG3'. Its frequence is lower than predictable by sequence. CpG dinucleotide are clustered in promoter regions of 50% of human genes (CpG Islands 0.5 – 5kb every 100kb). CpG islands are generally unmethylated in normal cells and their methylation determines downstream gene silencing. DNA methylation is brought about by a group of enzymes known as DNA methyltransferases (DNMT) and can be determined *de novo*, or be maintained from parental strain. Alterations of DNA methylation in cancer comprise global hypomethylation, dysregulation of DNA methyltransferase1, but, more frequently, regional hypermethylation in normally unmethylated CpG islands. Many genes have been demonstrated to be hypermethylated in neoplastic cells, belonging to different functional classes: for cell cycle regulation $p16^{ink4a}$, $p15^{ink4a}$,Rb,$p14^{arf}$, for DNA repair BRCA1, MGMT, for apoptosis DAPk,TMS1 and many others like ER, PR, e-cadherin, RARbeta. The mechanism by which DNA methylation achieves gene silencing is by interference with transcription factors binding at respective promoters and by regional recruitment of specific transcription repressors. Reversal of abnormalities in DNA methylation may restore expression of genes with tumor-suppressive function and provide a novel approach to cancer therapy. Two demethylating drugs, 5-azacytidine and 5-aza-deoxycytidine, are currently approved for clinical use, and several others are in preclinical development. We provide evidence for their activity *in vitro* on cell lines and primary human leukemic cells in modifying gene expression pattern and biological properties of transformed cells.

RELATIONSHIP BETWEEN MYELOMA CELLS AND THE BONE MICROENVIRONMENT: EFFECTS OF THE PROTEASOME INHIBITOR BORTEZOMIB.

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The bone microenvironment has a critical role in the pathogenesis and progression of multiple myeloma (MM).[1-2] Evidences show that both osteoclasts and osteoblasts may regulate MM cell growth and survival. Osteoclasts stimulate the proliferation of MM cells and support their survival mainly through the production of interleukin(IL)-6 and osteopontin.[3] In turn it is known that MM cells induce osteoclast formation and osteoclast bone resorbing activity leading to bone destruction.[3] The relationship between MM cells and osteoblasts is complex. Histomorphometric studies have demonstrated that, in MM patients with bone lesions, there is uncoupled or severely imbalanced bone remodeling with increased bone resorption and decreased or absent bone formation. In contrast, MM patients without bone lesions display balanced bone remodeling with increased osteoclastogenesis and normal or increased bone formation rates.[4-5] These histomorphometric studies are supported by clinical studies showing that MM patients with bone lesions have reduced bone formation markers, such as alkaline phosphatase and osteocalcin, together with the increased bone resorption markers.[6] Similarly, marked osteoblastopenia and reduced bone formation have also been reported in murine models of MM that develop bone lesions.[7] These data suggest that myeloma cells suppress osteoblasts and thereby inhibit bone formation. This inhibition of osteoblast activity plays a critical role in the pathogenesis of MM bone disease. On the other hand it has been demonstrated that osteoblastic cells are involved in the regulation of MM cell growth and survival. Osteoblasts secret IL-6 in co-culture systems with MM cells, contributing to the high IL-6 levels present in the bone marrow microenvironment that support MM cell growth.[8] In addition, osteoblastic cells may also stimulate MM survival by blocking TRAIL-mediated apoptosis of MM cells through the secretion of osteoprotegerin (OPG), a decoy receptor or both RANKL and TRAIL.[9] However, recently it has been reported that osteoblastic cells may attenuate the stimulatory effect of osteoclastic cells on MM cell survival,[10] suggesting a potential dual role of osteoblastic cells on the growth of MM cells dependent of the experimental conditions. Interestingly in the SCID-hu MM model, it was found that the stimulation of bone formation by the injection of mesenchymal stem cells resulted in an inhibition of MM growth suggesting that increasing bone formation in MM patients could result in a reduction in tumor burden.[10] The ubiquitin-proteasome pathway is the major cellular degradative system for several proteins involved in cell proliferation and survival in MM cells.[11] Recently, it has been demonstrated that this pathway may regulate osteoblast differentiation and bone formation *in vitro* and *in vivo* in mice.[12] The ubiquitin-proteasome pathway can modulate the expression of BMP-2,[12] which can induce osteoblast differentiation through the Wnt signaling and regulates the proteolytic degradation of the osteoblast transcription factor Runx2/Cbfa1.[13] Different proteasome inhibitors that bind the catalytic , subunits of the 20S proteasome and block its activity are able to stimulate bone formation in neonatal murine calvarial bones.[12] A strong correlation between the capacity of these compounds to inhibit proteasomal activity in osteoblasts and their bone forming activity was also demonstrated. Consistent with these in vitro observations, the administration of the natural proteasome inhibitors, PS1 and epoximicin, to mice increases bone volume and bone formation rate over 70% after 5 days,[12] indicating a potent stimulatory effect of these drugs on osteoblastic cells. The potential involvement of the ubiquitin-proteasome pathway in MM-induced osteoblast suppression is not known. However, these studies suggest a potential use of these drugs as anabolic agents in MM bone disease. This hypothesis is strongly supported by the *in vivo* observations obtained in MM patients treated with Bortezomib, the first representative of this class of drugs with a potent anti-myeloma activity actually recommended for the treatment of MM patients in relapse or non-responder ones and under investigation as first line therapy. An increase of total alkaline phosphatase and in parallel of bone specific alkaline phosphatase has been reported in MM patients that respond to the treatment with Bortezomib, but not in non-responders.[14-15] No data are available on the potential effect of Bortezomib on other markers of bone formation or osteolytic lesions in MM. However, based on results obtained in mice, Bortezomib may directly stimulate osteoblastic cells or osteoblast differentiation. Alternatively, the rapid apoptosis of MM cells induced by Bortezomib and their removal from the bone marrow could result in the recovery of osteoblast differentiation. Currently it is not know whether the proteasome inhibitor Bortezomib may have a direct effect on osteoblast and bone formation *in vitro* human cultures and *in vivo* in MM patients. To clarify this issue first we checked the effect of Bortezomib either on osteoblast differentiation and formation or on osteoblast proliferation, survival and function. In long-term human BM cultures we found that Bortezomib did not reduce the number of both early bone marrow (BM) osteoblast progenitors Colony Forming Unit-Fibroblast (CFU-F) and late ones Colony Forming Bone nodules (CFU-OB). On the other hand we found that Bortezomib significantly induced osteoblast phenotype in human mesenchymal cells incubated in presence of osteogenic factors. A stimulatory effect on osteoblast markers was observed after 24 hours of Bortezomib treatment. Consistently we found that Bortezomib significantly increased the activity of the transcription factor Runx2/Cbfa1 in human osteoblast progenitors without affecting the canonical WNT signaling pathway checked by the evaluation of nuclear and cytoplasmatic active beta-catenin levels. Using the human osteoblast like cells MG-63 and immortalized normal osteoblasts (HOBIT) we found that Bortezomib did not inhibit osteoblast proliferation or induce osteoblast apoptosis. Similarly, Bortezomib did not affect the expression of osteoblast markers, Runx2/Cbfa1 activity and WNT signaling in osteoblasts. To extent our *in vitro* observations we have evaluated the potential effect of Bortezomib *in vivo* in MM patients. Bone histomorphometry as well as immunostainig for Runx2/Cbfa1 and beta-catenin was performed on BM biopsies obtained from MM patients before and after 6-8 cycles of Bortezomib administrated in mono-therapy. A significant increase in the number of osteoblastic cells x mm^2 of bone tissue and in the number of Runx2/Cbfa1 positive osteoblastic cells was observed only in responder patients showing an early increase of the serum alkaline phosphatase. These data indicate that Bortezomib may increase osteoblast differentiation in human mesenchymal cells without affecting the proliferation, survival and function of mature osteoblasts. In vivo and in vitro observations support the hypothesis that both direct and indirect effects on bone formation process could occur during Bortezomib treatment.

References

1 Seidl S, Kaufmann H, Drach J. New insights into the pathophysiology of multiple myeloma. Lancet Oncol 2003;4:557-64.
2 Mitsiades CS, Mitsiades NS, Munshi NC, Richardson PG, Anderson KC. The role of the bone microenvironment in the pathophysiology and therapeutic management of multiple myeloma: Interplay of growth factors, their receptors and stromal interactions. Eur J Cancer. 2006 Jun 7; [Epub ahead of print].
3 Giuliani N, Colla S, Rizzoli V. New insight in the mechanism of osteoclast activation and formation in multiple myeloma: focus on the receptor activator of NF-kappaB ligand (RANKL). Exp Hematol. 2004;32:685-691.
4 Bataille R, Chappard D, Marcelli C, Dessauw P, Sany J, Baldet P, et al. Mechanisms of bone destruction in multiple myeloma: the importance of an unbalanced process in determining the severity of lytic bone disease. J Clin Oncol. 1989;7:1909-1914.
5 Bataille R, Chappard D, Marcelli C, Dessauw P, Sany J, Baldet P, et al. Osteoblast stimulation in multiple myeloma lacking lytic bone lesions. Br J Haematol. 1990;76:484-487.
6 Vejlgaard T, Abildgaard N, Jans H, Nielsen JL, Heickendorff L. Abnormal bone turnover in monoclonal gammopathy of undetermined significance: analyses of type I collagen telopeptide, osteocalcin, bone-specific alkaline phosphatase and propeptides of type I and type III procollagens. Eur J Haematol. 1997;58:104-108.
7 Hjorth-Hansen H, Seifert MF, Borset M, Aarset H, Ostlie A, Sundan A, et al. Marked osteoblastopenia and reduced bone formation in a model of multiple myeloma bone disease in severe combined immunodeficiency mice. J Bone Miner Res. 1999;14:256-263.
8 Karadag A, Oyajobi BO, Apperley JF, Russell RG, Croucher PI. Human myeloma cells promote the production of interleukin-6 by primary human osteoblasts. Br J Haematol. 2000;108:383-390.
9 Shipman CM, Croucher PI. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. Cancer Res. 2003;63:912-916.
10 Yaccoby S, Wezeman MJ, Zangari M, Walker R, Cottler-Fox M, Gaddy D, et al. Inhibitory effects of osteoblasts and increased bone formation on myeloma in novel culture systems and a myelomatous mouse model. Haematologica. 2006;91:192-199.
11 Kropff M, Bisping G, Wenning D, Berdel WE, Kienast J. Proteasome inhibition in multiple myeloma. Eur J Cancer. 2006 Jul 1; [Epub ahead of print]

12 Garrett IR, Chen D, Gutierrez G, et al. Selective inhibitors of the osteoblast proteasome stimulate bone formation in vivo and in vitro. J Clin Invest. 2003;111:1771-82.
13 Zhao M, Qiao M, Oyajobi BO, Mundy GR, Chen D. E3 ubiquitin ligase Smurf1 mediates core-binding factor alpha1/Runx2 degradation and plays a specific role in osteoblast differentiation. J Biol Chem. 2003;278:27939-44.
14 Zangari M, Esseltine D, Lee CK, Barlogie B, Elice F, Burns MJ et al. Response to bortezomib is associated to osteoblastic activation in patients with multiple myeloma. Br J Haematol. 2005;131:71-73.
15 Shimazaki C, Uchida R, Nakano S, Namura K, Fuchida SI, Okano A, et al. High serum bone-specific alkaline phosphatase level after bortezomib-combined therapy in refractory multiple myeloma: possible role of bortezomib on osteoblast differentiation. Leukemia. 2005;19:1102-3.

MOLECULAR MECHANISMS OF ARSENIC TRIOXIDE IN LEUKEMIA: SENSITIVITY AND DRUG-RESISTANCE

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Currently, Arsenic Trioxide (ATO) is considered the treatment of choice for patients with relapsed acute promyelocytic leukemia (APL), particularly in patients exposed to all-trans retionoic acid (ATRA) within the prior 12 months, replacing chemotherapy, ATRA or their combination as standard induction treatment in patients with relapsed APL.[1] Furthermore, a recent paper that reports a durable remission with minimal toxicity by single agent ATO in newly diagnosed APL is awakening great interest.[2] The results of clinical trials of ATO administration as single agent in other hematological malignancies such as multiple myeloma (MM) and myelodisplastic syndromes (MDS) are encouraging and show clinical effects but they are not close to APL success. On the contrary, results of clinical trials to treat non-APL acute myelogenous leukemia (AML) are disappointing.[3] We suggest that a combination therapy with drugs targeting specific pro-survival molecules or capable to enhance pro-apoptotic pathways may lead to an improvement of ATO efficacy against hematological malignancies by increasing drug-sensitivity and overcoming drug-resistance. The understanding of the mechanisms of ATO, in particular the changes in the activity of signaling proteins targeted by its administration could provide the rationale for combination therapies in order to increase its therapeutical efficacy against sensitive cells such as APL leukemic blasts, or to widen its spectrum to induce sensitivity in previously refractory or scarcely responsive tumor cells. In our laboratory we sought to understand which signaling pathways are involved to confer ATO resistance on acute leukemia cells. To perform these studies we isolated an arsenic-resistant NB4 subline (NB4-As$_R$) which showed stronger ERK1/2 activity (2.7 fold increase) and Bad phosphorylation (2.4 fold increase) compared to parental NB4 cells in response to ATO treatment. Upon ATO exposure, both NB4 and NB4-AsR cell lines doubled protein levels of the death antagonist Bcl-xL but the amount of free Bcl-xL that did not heterodimerize with phosphorylated Bad was 1.8 fold greater in NB4-AsR than in the parental line. A similar behaviour had Bcl-2. MEK1 inhibition down-modulated ERK activity, de-phosphorylated Bad and inhibited the ATO-induced increase of free Bcl-xL and Bcl-2, overcoming ATO resistance in NB4-AsR (4). Our results strikingly suggest that ATO is capable to induce cell death in acute leukemia cells but that its pro-apoptotic function is limited since it can induce also a mechanism of cell defense by activating pro-survival molecules such as MEK-ERK, Bcl-xL, Bcl-2.[4] The block of MEK-ERK phosphorylation and Bad de-phosphorylation interrupt the pro-survival mechanisms of ATO and kill the leukemic cells.[4]

These results prompted us to deepen the investigation of the mechanisms that regulate this special synergism. We found that in NB4 APL cells and in K562 erythroleukemia cell lines, bearing Bcr-Abl fusion protein, treatment with the MEK1 inhibitors PD98059 (Cell Signaling Technology, Beverly, MA) and PD184352 (kindly provided to us by Dr J. S. Sebolt-Leopold, Cancer Molecular Sciences, Pfizer Global Research & Development, Ann Arbor, MI) greatly enhanced apoptotic cell death induced by ATO alone. Combined treatment resulted in the induction of the p53AIP1 (p53-regulated Apoptosis-Inducing Protein 1) gene in both cell lines; p53AIP1 protein has been described to induce massive cell death.[5] Since NB4 and K562 cell lines carry an inactive p53 we investigated the possible role of p73, a p53 paralog that was shown to regulate several p53-target genes including p21, Bax and p53AIP1. We found that MEK1 inhibitors reduced the levels of dominant negative ΔN-p73 protein (anti-apoptotic and pro-proliferative) and promoted the accumulation of endogenous TAp73 (transactivation competent, pro-apoptotic and anti-proliferative) through its transcriptional activation and its tyrosine phosphorylation, resulting in p21 up-regulation and significant cell growth inhibition. ATO reduced ΔN-p73 levels and promoted a p300-mediated acetylation of endogenous p73, thus favouring cell cycle arrest and apoptosis.[6] Finally, the combined treatment with MEK1 inhibitors and ATO enhanced the affinity of phospho-acetylated p73 for the p53AIP1 promoter in vivo, as determined by chromatin immunoprecipitation experiments, leading to p53AIP1 up-regulation and increased apoptosis.[6]

Taken these values we tested in a pre-clinical setting whether MEK1 inhibition sensitizes different FAB subtypes of primary AML blasts to ATO-induced apoptosis.[7] We found that MEK1 inhibitor PD184352 strikingly increased apoptosis induced by ATO in 21 of 25 primary AML cases. Isobologram analysis confirmed the synergistic (13/25 cases) or additive (8/25 cases) nature of this interaction. Moreover, we demonstrated that the p53-related gene p73 is a molecular target of the combined treatment in AML blasts. Indeed, ATO modulated the expression of the p73 gene by inducing both the pro-apoptotic and anti-proliferative TAp73 and the anti-apoptotic and pro-proliferative ΔNp73 (this last behaviour is different from NB4 and K562 cell lines) isoforms thereby failing to elevate TA/ΔNp73 ratio. Conversely, the treatment with PD184352 reduced the level of ΔNp73 and blunted the arsenic-mediated up-regulation of ΔNp73, thus causing a raise of the TA/ΔNp73 ratio of dual-treated cells. Moreover, high doses of ATO induced p53 accumulation in 11/21 cases. Combined treatment resulted in the induction of the pro-apoptotic p53/p73-target gene p53AIP1, and greatly enhanced apoptosis of treated cells.[7] Furthermore, pre-treatment with MEK1 inhibitor strongly increased the expression of de-phosphorylated Bad and blunted the ATO-mediated phosphorylation of Bad at Ser112 in all the AML cases analyzed, thus confirming in primary AML blasts that this pathway may contribute, together with the p73-P53AIP1 pathway, to the induction of apoptosis in dual treated cells.[7]

These findings provide a rationale for an effective and relatively specific therapeutic strategy for AML to be tested in clinical trials.

References

1. Tallman MS, Nabhan C, Feusner JH, Rowe JM. Acute promyelocytic leukemia: evolving therapeutic strategies. Blood 2002; 99: 759-67.
2. Mathews V, George B, Lakshmi KM, Viswabandya A, Bajel A, Balasubramanian P et al. Single-agent arsenic trioxide in the treatment of newly diagnosed acute promyelocytic leukemia: durable remissions with minimal toxicity. Blood 2006; 107: 2627-32.
3. Amadori S, Fenaux P, Ludwig H, O'dwyer M, Sanz M. Use of arsenic trioxide in haematological malignancies: insight into the clinical development of a novel agent. Curr Med Res Opin 2005; 21: 403-11.
4. Lunghi P, Tabilio A, Lo Coco F, Pelicci PG, Bonati A. Arsenic Trioxide (ATO) and MEK1 inhibition synergizes to induce apoptosis in acute promyelocytic leukemia cells. Leukemia 2005. 19: 234-44.
5. Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T et al. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell 2000; 102: 849-862.
6. Lunghi P, Costanzo A, Levrero M, Bonati A. Treatment with Arsenic Trioxide (ATO) and MEKl inhibitor activates the p73-p53AIPl apoptotic pathway in leukernia cells. Blood 2004; 104: 519-25.
7. Lunghi P, Costanzo A, Salvatore L, Noguera N, Mazzera L, Tabilio A et al. MEK1 inhibition sensitizes primary acute myelogenous leukemia to arsenic trioxide-induced apoptosis. Blood 2006; 107:4549-53.

MOLECULAR BASIS OF PHILADELPHIA-NEGATIVE CHRONIC MYELOPROLIFERATIVE DISORDERS

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The identification of a unique gain-of-function mutation of the Janus kinase 2 (*JAK2*) gene in patients with chronic myeloproliferative disorders[1-4] clearly represents a major breakthrough in the pathogenesis of these conditions. An article summary in the New England Journal of Medicine (Apr 28, 2005) stated that this identification opened new avenues of research, which could lead to improved diagnosis and treatment. In effect, in less than ten months an impressive number of papers have appeared that not only confirm the initial findings but also provide novel observations of biologic and clinical interest.

By using allele-specific PCR, the frequency of the *JAK2* (V617F) mutation has been found to be greater than 90% in polycythemia vera, greater than 50% in essential thrombocythemia and around 50% in chronic idiopathic myelofibrosis. The majority of patients with polycythemia vera are therefore positive for *JAK2* (V617F), which should definitely be considered as a major diagnostic criterion for this condition. However, it should also taken into account that there is a minority of patients with polycythemia vera, both sporadic and familial cases, in whom the mutation is absent.

Several observations suggest that *JAK2* (V617F) participates in the pathogenesis of myeloproliferative disorders.[5] In particular, recent studies have shown that the expression of the mutant gene in murine hematopoietic cells leads to myeloproliferative disorders mimicking human PV with secondary myelofibrosis.[6,7]

A study[8] has likely clarified why the mutation is found in clonal disorders of myeloid, but not lymphoid, lineage and why it is mainly present in myeloid-lineage cells in myeloproliferative disorders. JAK2 plays a central role in the signal transduction by homodimeric type-I cytokine receptors such as the erythropoietin receptor, granulocyte colony-stimulating-factor receptor or thrombopoietin receptor, and signal transduction by JAK2 (V617F) requires a cytokine receptor as a scaffold. While myeloid-lineage cells express these type-I cytokine receptors, lymphoid-lineage cells do not. This clarifies why the occurrence of the JAK2 (V617F) mutation in a multipotent hematopoietic stem cell results in a selective expansion of its myeloid-lineage cell progeny.

A two-step model[4] for the role of *JAK2* (V617F) explains the clonal evolution of myeloproliferative disorders. The first step consists of a G-to-T transversion that changes a valine to a phenylalanine at position 617 in one *JAK2* allele on chromosome 9p: this gives rise to a clone that is heterozygous for *JAK2* (V617F) and expands to replace hematopoietic cells without the *JAK2* mutation. The second step consists of a mitotic recombination in a hematopoietic cell that is heterozygous for *JAK2* (V617F): this generates uniparental disomy and loss of heterozygosity of 9p (9pLOH) in one of the two daughter cells. The daughter cell that is homozygous for *JAK2* (V617F) gives rise to a new clone that expands and replaces the previous heterozygous clone (clonal dominance). Both the transition from normal hematopoiesis to a clonal population of heterozygous cells, and that from heterozygous to homozygous clonal dominance are continuous processes characterized by mixed populations of cells. The available evidence suggests that this model might best apply to polycythemia vera, where the vast majority of patients carry the mutation.

We previously concluded that gain of function and loss of control appear to be the essential features of the excessive myeloproliferation associated with *JAK2* (V617F).[5] Gain of function results in increased production of mature blood cells. Loss of control means that the normal mechanisms that regulate circulating cell counts (for instance, transcriptional feedback regulation by erythropoietin) are no longer effective. In addition, loss on control means that activated mature blood cells – the progeny of expanded hematopoietic cells – have major effects on disease phenotype.

We recently reported that patients with myeloproliferative disorders have granulocyte activation patterns similar to those induced by granulocyte colony-stimulating factor.[9,10] These patients also have variably elevated circulating CD34-positive cell counts. A *JAK2* (V617F) gene dosage effect was observed on both CD34-positive cell counts and granulocyte activation in polycythemia vera, where abnormal patterns were mainly found in patients carrying >50% mutant alleles. These observations suggest that *JAK2* (V617F) may constitutively activate granulocytes and by this means mobilize CD34-positive cells.

These observations not only contribute to clarify the pathophysiology of myeloproliferative disorders, but also provide useful diagnostic and prognostic information. For instance, sequential evaluation of the percentage of JAK2 mutant alleles and enumeration of circulating CD34-positive cells appear potentially very useful for disease monitoring in polycythemia vera.

References

1. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 2005;365:1054-61.
2. Levine RL, Wadleigh M, Cools J, Ebert BL, Wernig G, Huntly BJ, et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005;7:387-97.
3. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature 2005;434:1144-8.
4. Kralovics R, Passamonti F, Buser AS, Teo SS, Tiedt R, Passweg JR, et al. A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 2005;352:1779-90.
5. Cazzola M, Skoda R. Gain of function, loss of control - a molecular basis for chronic myeloproliferative disorders. Haematologica 2005;90:871-4.
6. Wernig G, Mercher T, Okabe R, Levine RL, Lee BH, Gilliland DG. Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. Blood 2006;107:4274-81.
7. Lacout C, Pisani DF, Tulliez M, Moreau Gachelin F, Vainchenker W, Villeval JL. JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. Blood 2006;[Epub ahead of print].
8. Lu X, Levine R, Tong W, Wernig G, Pikman Y, Zarnegar S, et al. Expression of a homodimeric type I cytokine receptor is required for JAK2V617F-mediated transformation. Proc Natl Acad Sci USA 2005;102:18962-7.
9. Kralovics R, Teo SS, Buser AS, Brutsche M, Tiedt R, Tichelli A, et al. Altered gene expression in myeloproliferative disorders correlates with activation of signaling by the V617F mutation of Jak2. Blood 2005;106:3374-6.
10. Passamonti F, Rumi E, Pietra D, Della Porta MG, Boveri E, Pascutto C, et al. Relation between JAK2 (V617F) mutation status, granulocyte activation, and constitutive mobilization of CD34+ cells into peripheral blood in myeloproliferative disorders. Blood 2006;107:3676-82.

THROMBOSIS IN ONCO-HEMATOLOGIC MALIGNANCY

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Onco-hematologic malignancies impose an inherent risk of thrombosis that is increased by chemotherapy and other risk factors. Multiple myeloma (MM) and other plasma cell dyscrasias are thrombogenic as a consequence of their multiple haemostatic effects. The oral drugs thalidomide and lenalidomide have produced major therapeutic responses in patients with MM when used in combination with oral steroids and chemotherapy, but significantly increased the risk of venous thromboembolism (VTE).[1] In newly diagnosed patients receiving thalidomide in combination with chemotherapy, prophylactic low-dose warfarin sodium was shown to reduce VTE rate to levels seen without thalidomide.[2] Non-Hodgkin and Hodgkin lymphomas also carry a significant risk for venous and arterial thrombosis, particularly during chemotherapy treatments.[3,4] Hemostatic alterations underlying a hypercoagulable condition are commonly found in patients with lymphomas as well as in all other haematological malignancies.[5]

In this setting, acute leukaemia represents a unique model, as it shows the entire variety of clinical and laboratory signs of malignancy-associated coagulopathy. Clinical manifestations due to different degrees of disseminated intravascular coagulation (DIC) can range from bleeding to thrombosis of large vessels. The risk is different according to: 1. the type of leukaemia, i.e.: acute myeloblastic leukemia (AML), acute lymphoblastic leukemia (ALL), or acute promyelocytic leukemia (APL); and 2. the phase of treatment, i.e.: onset of the disease, remission induction, consolidation. Thrombosis has been considered rarer than bleeding in adult acute leukaemias, however recent data indicate that it is a relevant problem.[6] A recent prospective study demonstrated a thrombosis rate of 3.2% in AML (excluding APL) versus 1.4% in ALL at diagnosis, and of 1.7% in AML versus 10.6% in ALL during induction chemotherapy.[7] Similar data were obtained in patients prospectively followed at our centre from 2000 to 2006 and confirmed that the thrombosis rate at the onset is greater in AML than in ALL, while in induction is greater in ALL.

APL is the M3 subtype of AML, which prominently presents with bleeding symptoms, but also thrombosis can be seen.[7] In a prospective cohort of 42 APL patients at our centre, 4.7% had thrombosis at diagnosis and 9.5% during induction therapy. Thrombotic complications typically occurred in conjunction with bleeding symptoms as a part of the thrombo-haemorrhagic diathesis of these patients. Before the introduction of all-trans retinoic acid (ATRA) in the management of APL, fatal haemorrhages were a major cause of induction remission failure.[8] ATRA has produced a high rate of CR and a rapid resolution of the coagulopathy,[9] although early fatal haemorrhages still range between 2.4% and 6.5%.

Major determinants for the pathogenesis of the coagulopathy of acute leukaemia are: 1. Factors associated with leukaemic cells, i.e. the expression

of procoagulant, fibrinolytic and proteolytic properties, and the secretion of inflammatory cytokines, 2. Cytotoxic therapies; and 3. Concomitant infectious complications. Many studies have characterised the procoagulant activities (PCA) expressed by leukaemic cells, particularly 'tissue factor' (TF) and 'cancer procoagulant' (CP).[10] AML and ALL blasts express significant amount of PCA, with the greatest expression in APL cells. Laboratory abnormalities of the blood clotting system underlying the clinical pictures of DIC are observed in both AML and ALL,[11] and worsen upon initiation of chemotherapy. Routine test alterations include hypofibrinogenemia, increased FDPs and prolonged prothrombin and thrombin times. These abnormalities reflect the activation of both coagulation, fibrinolysis and non-specific proteolysis. Studies of hypercoagulation markers clearly show that thrombin generation constantly occurs in acute leukaemia. Particularly, the increase of D-dimer, the by-products of cross-linked fibrin, demonstrates ongoing hyperfibrinolysis in response to clotting activation.[9,10] The advent of ATRA for the remission induction therapy of APL has opened new perspectives in the management of the coagulopathy. Different laboratories, including ours, have shown the decrease or normalization of clotting and fibrinolytic variables during the first one or two weeks of ATRA therapy.[10,12] The benefical effect on laboratory parameters is associated with the improvement of clinical signs of the coagulopathy. In our study of 42 APL patients we found, before therapy, elevated levels of FVIIa-Antithrombin complex, as an expression of increased TF activity in the blood, and elevated TF pathway inhibitor (TFPI). After starting ATRA, FVIIa-Antithrombin complex dropped within 7-15 days in parallel to TFPI and to all markers of clotting activation (TAT, F1+2, D-dimer). This demonstrates that ATRA reduces TF-induced clotting activation *in vivo*. Thrombotic complications can affect morbidity and even mortality in these patients. No ad hoc studies or guidelines are available for prophylaxis or treatment of VTE in onco-hematological malignancies. The use of low molecular weight heparins (LMWH) has improved VTE treatment in patients with solid tumours, but no experience has been accumulated in patients with acute leukaemia, who carry a significant risk of hemorrhage, due to the profound and prolonged cytopenias secondary to intensive chemotherapy regimens. These limitations increase the interest in preventing VTE occurrence in these patients.

References

1. Barbui T, Falanga A. Thalidomide and thrombosis in multiple myeloma. J Thromb Haemost. 2003; 1:421-2.
2. Cavo M, Zamagni E, Cellini C, Tosi P, Cangini D, Cini M et al. Deep vein thrombosis in patients with multiple myeloma receiving first-line thalidomide-dexamethasone therapy. Blood 2002; 100: 2273.
3. Khorana AA, Francis CW, Culakova E, Lyman GH. Risk factors for chemotherapy-associated venous thromboembolism in a prospective observational study. Cancer 2005; 104: 2822-9.
4. Khorana AA, Francis CW, Culakova E, Fisher RI, Kuderer NM, Lyman GH. Thromboembolism in hospitalized neutropenic cancer patients. J Clin Onc 2006; 24: 484-490.
5. Barbui T, Finazzi G, Falanga A: The management of bleeding and thrombosis in acute leukemia and chronic myeloproliferative disorders. Leukemia (7th edition). ES. Henderson, TA Lister, MF Greaves (Eds.), WB Saunders Company 2001, Chapter 17.
6. Zigler S, Sperr WR, Knobl P, Weltermann A, Jager, et al. Symptomatic venous thromboembolism in acute leukemia. Incidence, risk factors, and impact on prognosis. Thromb Res. 2005; 115: 59-64.
7. De Stefano V, Sora F, Rossi E, Chiusulo P, Laurenti L, Fianchi L, et al. The risk of thrombosis in patients with acute leukemia: occurrence of thrombosis at diagnosis and during treatment. J Thromb Haemost. 2005; 3:1985-92.
8. Rodeghiero F, Avvisati G, Castaman G, Barbui T, Mandelli F. Early deaths and anti-hemorrhagic treatments in acute promyelocytic leukemia. A GIMEMA retrospective study in 268 consecutive patients. Blood 1990; 75: 2112-17.
9. Falanga A, Rickles FR. Pathogenesis and management of the bleeding diathesis in acute promyelocytic leukaemia. Best Pract Res Clin Haematol. 2003;16(3):463.
10. Falanga A, Iacoviello L, Evangelista V, Belotti D, Consonni R, D'Orazio A, Robba L, Donati MB, Barbui T. Loss of blast cell procoagulant activity and improvement of hemostatic variables in patients with acute promyelocitic leukemia given all-trans-retinoic acid. Blood 1995;86:1072-1084.
11. Leone G, Gugliotta L, Mazzucconi MG, et al: Evidence of a hypercoagulable state in patients with acute lymphoblastic leukemia treated with low dose of E. coli L-Asparaginase: a GIMEMA study. Thromb Haemost 1993; 69: 12.
12. Tallman M.S, Lefebvre P, Baine R.M, et al: Effects of all-trans retinoic acid or chemotherapy on the molecular regulation of systemic blood coagulation and fibrinolysis in patients with acute promyelocitic leukemia. Journal of Thrombosis and Haemostasis 2004; 2:1341.

TISSUE MICRO-ARRAYS IN HAEMATOPATHOLOGY: PRESENT USAGE AND POTENTIAL

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The tissue micro-array technology represents a novel tool for clinico-pathologic studies, that allows the rapid, chip and efficient analysis of large series of cases. In particular, it represents the natural integration of gene expression profiling techniques as to what their proteomic validation is concerned.

The tissue micro-array (TMA) technology (Figure 1) was originally developed at the Institute of Pathology of Basel University[1] and has subsequently found extensive application to haematopoietic tumour analysis.[2-6] It consists in punching routinely-fixed paraffin-embedded tissue blocks by a needle measuring 0.6-2 mm across in areas previously identified on conventionally stained preparations, obtained from the same blocks and representative of the process under investigation. Two-three tissue cores from the same case are moved to a newly created paraffin block that contains up to 1,000 cores. Sections cut from the latter allow the simultaneous analysis of hundreds of cases by means of immunohistochemistry or fluorescence in situ hybridization (FISH). On average, 50-60 sections can be obtained from each TMA. The main benefits offered by such approach are: 1) preservation of the archive material that is only minimally used for TMA construction, 2) simultaneous and optimised analysis of a large series of cases on the same section, and 3) spare of time and money. In fact, the expression of dozens of molecules can be assessed on a limited number of sections by using small aliquots of expensive reagents and lowering both the technical and medical workload. The evaluation of the results obtained may be facilitated by TMA digitalisation and computer-assisted analysis.

Comparative studies have shown that TMA efficiency is indeed high: in fact, although some loss of cores is expected, at least 90% and 50% of the cases are fully evaluated by means of immunohistochemical and FISH techniques, respectively.[2,5]

TMAs are an invaluable tool when one aims to check the expression of molecules of potential prognostic and/or therapeutic relevance. In fact, the results on TMAs can be matched with the response to therapy and follow-up data in the course of retrospective and prospective studies. In addition, they can be easily employed for the validation of gene expression profiling studies at the protein level. In particular, the products of genes found to be systematically deregulated in small series of samples with optimally preserved m-RNA can be searched on large routine-tissue collections contained on the same TMA.

Such tchnique has been used by our Group in the course of several studies. First of all, we tried to surrogate the results of gene expression profiling in the field of diffuse large B-cell lymphoma. By the application of 7 markers (i.e. CD10, Bcl-2, Bcl-6, IRTA-1, CD30, IRF4/MUM-1, and CD138) to TMAs from 68 patients staged II – IV and treated with MACOP-B, three groups of tumours were identified.[7] Thirty-six cases corresponded to Bcl-2-negative germinal centre cell-derived lymphomas with low IPI (0.6) and prolonged 3-yr-overall (OS: 91%) and relapse-free survival (DFS: 86%), 17 had an activated phenotype with high IPI (1.9) and dismal prognosis (OS: 38%, RFS: 41%), and 15 showed an undetermined profile with intermediate IPI (1.1) and behaviour (OS: 66%, RFS: 63%). Secondly, we tested the feasibility of the ZAP-70 immunohistochemical test on bone-marrow biopsies (BMBs) and compared the results with those by Western Blotting (WB) and IgV_H Polymerase Chain Reaction (PCR) in the peripheral blood.[8] For this purpose, 26 CLL/SLL patients were selected with onset BMB and IgV_H mutational status. ZAP-70 was immunohistochemically determined by three different antibodies (from Upstate, Cells Signalling, and Santa Cruz respectively) and two different methods (APAAP and EnVision+). In 23 cases, ZAP-70 WB results were also available. ZAP-70 determination on BMBs turned out easily feasible with routine procedures with reagents from Upstate and Cell Signalling. The results were concordant with those in PCR and WB in 84.9% and 82.6% respectively. Three of four discordant cases were mutated/ZAP-70 positive with weak staining for ZAP-70 in two both at WB and IHC. Finally, we validated the results obtained by gene expression profiling in 17 peripheral T-cell lymphomas of the unspecified type (PTCL/U) by testing TMAs of 193 PTCLs with a large panel of antibodies corresponding to deregulated gene products. Such strategy allowed, on one hand, the identification of a new mixed clinico-pathologic prog-

nostic score,[9] on the other the confirmation - on tissue samples - of the PDGFRα over-expression recorded at genomic analysis.[10] Notably, the latter finding has potential therapeutic implications (i.e. the usage of tyrosine-kinase inhibitors for the treatment of PTCLs).

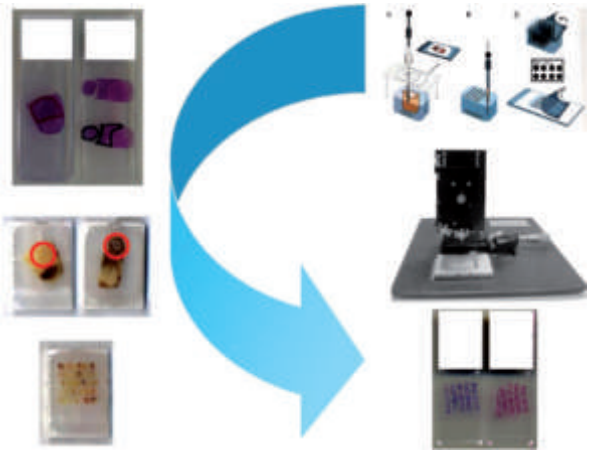

Figure 1. Schematic representation of the tissue micro-array technology.

We declare that all the experiments on human subjects were conducted in accordance with the Helsinki Declaration. This paper does not overlap with any other publication. None of the authors have any conflict of interest to declare.

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References

1. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844-7.
2. Pileri SA, Dirnhofer S, Went P, Ascani S, Sabattini E, Marafioti T, et al. Diffuse large B-cell lymphoma: one or more entities? Present controversies and possible tools for its subclassification. Histopathology 2002;41:482-509.
3. Tzankov A, Zimpfer A, Pehrs AC, Lugli A, Went P, Maurer R, et al. Expression of B-cell markers in classical hodgkin lymphoma: a tissue microarray analysis of 330 cases. Mod Pathol 2003;16:1141-7.
4. Tzankov A, Pehrs AC, Zimpfer A, Ascani S, Lugli A, Pileri S, et al. Prognostic significance of CD44 expression in diffuse large B cell lymphoma of activated and germinal centre B cell-like types: a tissue microarray analysis of 90 cases. J Clin Pathol 2003;56:747-52.
5. Tzankov A, Zimpfer A, Lugli A, Krugmann J, Went P, Schraml P, et al. High-throughput tissue microarray analysis of G1-cyclin alterations in classical Hodgkin's lymphoma indicates overexpression of cyclin E1. J Pathol 2003;199:201-7.
6. Zimpfer A, Schonberg S, Lugli A, Agostinelli C, Pileri SA, Went P, et al. Construction and validation of a bone marrow tissue microarray. J Clin Pathol 2006.
7. Zinzani PL, Dirnhofer S, Sabattini E, Alinari L, Piccaluga PP, Stefoni V, et al. Identification of outcome predictors in diffuse large B-cell lymphoma. Immunohistochemical profiling of homogeneously treated de novo tumors with nodal presentation on tissue micro-arrays. Haematologica 2005;90:341-7.
8. Sabattini E, Orduz, R., Campidelli, C., Zinzani, PL, Callea V, Zupo, et al. B-cell chronic lymphocytic leukaemia/small lymphocytic lymphoma: role of ZAP-70 determination on bone marrow biopsies. Journal of Clinical Pathology;In press.
9. Went P, Agostinelli C, Gallamini A, Piccaluga PP, Ascani S, Sabattini E, et al. Marker expression in peripheral T-cell lymphoma: a proposed clinical-pathologic prognostic score. J Clin Oncol 2006;24:2472-9.
10. Piccaluga PP, Agostinelli C, Zinzani PL, Baccarani M, Dalla Favera R, Pileri SA. Expression of platelet-derived growth factor receptor alpha in peripheral T-cell lymphoma not otherwise specified. Lancet Oncol 2005;6:440.

MINIMAL RESIDUAL DISEASE AND ALLOGENEIC STEM CELL TRANSPLANTATION

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Introduction. Relapse remains a significant issue in acute leukemia patient undergoing an allogeneic hemopoietic stem cell transplant (HSCT). Once a patient with acute leukemia has a hematologic relapse post-transplant, cell therapy, such as donor lymphocyte infusions (DLI) is usually ineffective and survival is poor. This is in contrasts with the strong graft versus leukemia (GvL) effect exherted by chronic graft versus host disease (GvHD) , in patients with acute leukemia. Perhaps, if we could detect recurrence before a full blown hematologic relapse is evident, then immune intervention may be more effective.

Patients and methods. To test this hypopthesi we prospectively monitorede minimal residual disease (MRD) in 80 patients with acute lymphoid (ALL, N=44) or myeloid (AML, n=36) leukemia, undergoing an allogeneic hemopoietic stem cell transplant. MRD markers were IgH-VDJ and TCR gene re-arrangement for ALL and Wilms Tumour (WT1) expression for AML.

Results. The overall cumulative incidence (CI) of MRD positivity was 45% and the CI of hematologic relapse was 22% (36% in MRD+ vs 11% in MRD- patients, p=0.008). The median interval from transplant to first MRD positivity was day +120, and to hematologic relapse, day +193. Patients could be divided in 3 MRD groups: MRD- (n=44), MRD+ given donor lymphocyte infusions (DLI) (n=17) and MRD+ not given DLI (n=19): leukemia relapse in these 3 groups was respectively 11%, 6% and 63% (p=0.0005); the actuarial 3 year survival was 92%, 80% and 26% (p=0.0006). In multivariate COX analysis, MRD group predicted relapse (p=0.0008) and survival (p=0.003), together with disease phase and chronic GvHD. In MRD+ patients, DLI protected against relapse (p=0.003) and improved survival (p=0.01).

Conclusions. (a) MRD positivity post-transplant predicts hematologic relapse in patients with acute leukemia. (b) MRD positivity anticipates relapse by a median of 73 days. (c) DLI protects MRD+ patients against relapse, and produces survival comparable to MRD- patients. This suggests GvL can be adoptively transferred in patients with acute leukemia and minimal residual disease.

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CLINICAL VALUE OF MINIMAL RESIDUAL DISEASE (MRD) MONITORING IN LYMPHOMA PATIENTS UNDERGOING AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Current treatment strategies for non-Hodgkin's lymphoma include intensive treatment protocols with autologous stem cell transplantation (ASCT). High-dose therapy with ASCT as part of first-line treatment induce marked tumor reduction leading to a substantial prolongation of disease-free (DFS) and overall survival, at least in younger patients with high-risk Follicular (FL) or Mantle-Cell Lymphoma (MCL).[1] Following the pioneering work of the Dana Farber group, the effectiveness of the treatment can now be controlled at a very high level of sensitivity and accuracy by molecular monitoring of minimal residual disease (MRD) during and after treatment.[2] MRD analysis is based on polymerase chain reaction (PCR) amplification of clone-specific immunoglobulin heavy chain (IGH) gene rearrangements or fusion genes (BCL2-IGH in FL, BCL1-IGH in MCL), and allows the detection of one single tumor cell among 10^5 normal cells in circulation or bone marrow.[2,3].

Several years ago, an innovative high-dose approach, including peripheral blood progenitor cell (PBPC) autograft, has been developed as first-line treatment for indolent lymphoma.[4] The approach proved to be effective, allowing prolonged survival, with durable response in most FL patients. Since the initial experience, molecular MRD analysis was employed as essential tool for monitoring response to treatment. Recently, the MRD studies have been updated after a long follow-up.[5] The results indicate that: i. a molecular marker can be obtained in approximately 85% of patients with indolent B-cell lymphoma; ii. the collection of PCR-negative PBPC harvests is feasible through the in vivo purging effect operated by intensive chemotherapy, without the need of *ex-vivo* purging procedures; iii. the achievement of post-ASCT molecular remission is more frequently achieved in FL subtype and is predictive for a

prolonged progression-free survival (PFS), while persistent PCR positivity is associated to a high relapse risk. This latter observation is in line with the Dana Farber reports, showing that detection by PCR of MRD was associated with increased risk of relapse, after ASCT with *ex-vivo* purged bone marrow cells.[2] More recent studies have confirmed that MRD is a critical factor also in MCL, influencing the prognosis of patients after front-line high-dose treatment and ASCT.[6] Based on the promising results of the single Center experience, a multicenter, prospective trial was launched in 1996 by 20 hematological Centers affiliated to the Gruppo Italiano Trapianto Midollo Osseo (GITMO) to evaluate applicability and efficacy of the intensified high-dose chemotherapy program as front-line therapy in 92 high-risk FL patients.[7] Centralized molecular analysis showed that PCR-negative harvests could be collected in 47% of cases. Following autograft, 65% of molecularly evaluable patients achieved clinical and molecular remission: the projected DFS at five years of this subgroup is 85%. The result emphasizes the importance of achieving maximal tumor reduction in FL patients. In addition, the multicenter trial showed that highly effective intensified treatments can now be routinely offered to young patients with poor risk FL even at small Institutions, with no need for sophisticated and expensive cell manipulation procedures. The outcome of B-cell Lymphoma patients has definitely improved since the introduction of the anti-CD20 Rituximab, which can be effectively combined into conventional chemotherapy. Indeed, molecular remission has been observed in FL patients following Rituximab-supplemented CHOP (CHOP-R).[8] Rituximab may also improve ASCT-based programs, particularly as an *in vivo* purging agent prior to PBPC harvests.[1] Thus, a randomized GITMO trial has been recently performed comparing CHOP-R vs. Rituximab-supplemented intensive program in high-risk FL 9. Preliminary results of MRD analysis indicate a higher frequency of molecular remission in the intensive program arm compared to CHOP-R (78% vs. 26%, respectively; p<0,001). The achievement of a persistent molecular remission was associated to an improved PFS; interestingly, PFS of PCR-positive and PCR-negative patients was analogous in the two treatment arms.

In conclusion, the results presented demonstrate that MRD monitoring is a powerful indicator of treatment outcome in patients with NHL undergoing high-dose treatment and ASCT, with or without Rituximab administration. MRD elimination after ASCT is highly predictive of a favorable prognosis, making molecular remission a major goal in this treatment setting. Although many patients achieving molecular remission experience a prolonged DFS, molecular relapse can still occur and may represent an early warning for subsequent clinical relapse. We have recently reported on patients experiencing molecular relapse following a documented molecular response achieved with a Rituximab-supplemented high-dose program.[10] Patients were re-treated with Rituximab and they all re-entered molecular remission. This further substantiate the clinical value of MRD monitoring in lymphoma patients during and after intensive treatments with autograft.

References

1. Tarella C, Gianni AM. BMT for Lymphoma CR1. Curr Opin Oncol 2005; 17: 99-105.
2. Gribben JG, Nadler LM. Monitoring minimal residual disease. Semin Oncol. 1993; 20 (Suppl 5):143-55
3. Corradini P, Ladetto M, Pileri A, Tarella C. Clinical relevance of minimal residual disease monitoring in non-Hodgkin's lymphomas: a critical reappraisal of molecular strategies. Leukemia 1999; 13: 1691-5
4. Tarella C, Caracciolo D, Corradini P, et al. Long-term follow-up of advanced-stage low-grade lymphoma patients treated upfront with high-dose sequential chemotherapy and autograft. Leukemia. 2000; 14: 740-7
5. Corradini P, Ladetto M, Zallio F, et al. Long-term follow-up of indolent lymphoma patients treated with high-dose sequential chemotherapy and autografting: evidence that durable molecular and clinical remission frequently can be attained only in follicular subtypes, J Clin Oncol 2004; 22: 1460-68
6. Pott C, Schrader C, Gesk S, et al. Quantitative assessment of molecular remission after high-dose therapy with autologous stem cell transplantation predicts long-term remission in mantle cell lymphoma. Blood 2006; 107: 2271-78
7. Ladetto M, Corradini P, Vallet S, et al. High rates of clinical and molecular remissions in Follicular Lymphoma patients receiving high-dose sequential chemotherapy and autografting at diagnosis: a multicenter, prospective study by the Gruppo Italiano Trapianto Midollo Osseo (GITMO). Blood 2002; 100: 1559-65
8. Rambaldi A, Lazzari M, Manzoni C, et al. Monitoring of minimal residual disease after CHOP and rituximab in previously untreated patients with follicular lymphoma, Blood 2002; 99: 856-62
9. Tarella C, Ladetto M, Vallet S, De Marco F, Corradini P, a nome del GITMO. Linfomi Follicolari ad alto rischio: Studi GITMO. Atti, 40° Congresso Nazionale SIE, Bergamo 2005, pag. 190-199
10. Ladetto M, Magni M, Pagliano G, et al. Rituximab induces effective clearance of Minimal Residual Disease in Molecular Relapses of Mantle Cell Lymphoma Biol Blood Marrow Transpl, in press

INNOVATIVE CELLULAR THERAPIES

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Cytokine induced killer cells (CIK) are CD3+/CD56+ cells expanded in vitro with rhIL-2 after stimulation of peripheral blood mononuclear cells with OKT3 and IFN- . They are T/NK cells with cytotoxic potential against leukemic and other tumor cells but not normal bone marrow in vitro and in vivo. We have performed a phase I study with allogeneic (donor's) CIK cells given to patients relapsing after allogeneic bone marrow transplantation and show evidence of anti-leukemic activity in vivo with very little GVHD. Eleven patients with AML (n=4), HD (n=3), CMML (n=1), pre-B ALL (n=1) and MDS (n=2), all relapsed after sibling (6) or matched unrelated donor (5) HSCT, entered this study. The median number of CIK infusions was 2 (range 1-7) and the median number of total CIK cells was $14.5x10^6$/kg (range 7.2-51). The infusions were well tolerated and no acute or late infusion-related reactions were registered. Acute GVHD (grade I and II) was observed in 4 patients, which progressed into extensive chronic GVHD in 2 cases. In 6 patients, no significant clinical response could be registered so that disease progression and death occurred rapidly. Five patients achieved measurable responses: one patient with MDS, who had been treated with CIK cells alone, showed a hematologic improvement but subsequently progressed and died. One patient with HD received local radiotherapy followed by 7 CIK infusions (total of $51x10^6$/kg CIK cells) which allowed the achievement of a good PR. After almost 1 year, he progressed and chemotherapy was given with achievement of a very good PR. A second patient with HD received one DLI at day 516 and 1 CIK infusion ($12.2x10^6$/kg) at day 537. At day 572, chemotherapy was initiated due to the persistence of disease. At the end of chemotherapy he received 3 additional CIK infusions for a total of $34x10^6$/kg from days 711-752, and is presently in CR at more than 780 days. One patient with CMML had been treated with DLI on day 102 and with $38x10^6$/kg CIK cells, given in four infusions from days 137-530, because of the appearance of mixed chimerism. Full chimerism was achieved 42 days after the first CIK infusion. The patient remains in CR at day 576. A second MDS patient, who had not achieved any significant response after five DLI (days 411-559), obtained a complete hematologic, cytogenetic and molecular remission after a single CIK infusion ($7.6 x10^6$/kg) given at day 603 and remains in CR at day 680. Cord blood (CB) transplantation is progressively becoming an extensively used treatment for patients with malignant disorders. One major limitation of this procedure is the lack of donor derived cells to perform DLI in case of relapse. In order to extend the use of CIK cells to the CB transplantation setting, we have standardised a 21 days expansion protocol to produce CIK cells starting from very small amounts of nucleated cells isolated from cord blood.. Three used CB bags were returned to the laboratory after transplantation and repeatedly washed. An average of 22 $x10^6$ nucleated cells could be recovered, yielding a mean 473 $x10^6$ CD3/CD56+ cells at the end of the culture period. CIK cells generated from CB showed strong cytotoxic activity against a variety of tumor target cell lines including B and T lymphomas and myeloid leukemias. More importantly, they were cytotoxic against AML blasts isolated from 2 patients. During expansion CB derived CIK cells upregulated the NKG2D marker and expressed perforin and granzyme molecules in >90% of cells. These observations open up the possibility of a future clinical application of this protocol, performed in GMP conditions. Patients relapsing following cord blood transplantation may be treated with CIK cells expanded from the same cord blood unit, where donors would not be anymore available for cell mediated immunotherapy.

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TUMOR IDIOTYPE AS ANTIGENE TARGET IN MULTIPLE MYELOMA

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Early during development, pre-B cells become committed to the expression of a heavy and light chain Ig variable region. The heavy chain derives from the recombination of a variable (V) with a diversity (D) and a joining (J) region genes with a constant region (C). The V-D-J joints occur with a variable number of nucleotide insertions or deletions resulting in a unique sequence which creates the third hypervariable region (CDR III) and contributes to the antigen binding site. These antigenic regions (idiotype; Id) are characteristic for any given Ig producing tumor (i.e. multiple myeloma, MM; non-Hodgkin lymphoma; NHL) and can be recognized by an immune response consisting of anti-Id antibodies and/or by Id reactive T-cells. The tumor derived Id is a *self protein* which is in most circumstances poorly immunogenic. However, it can be made immunogenic if it is coupled to a carrier protein and administered in an immunological adjuvant formulation. Among professional antigen presenting cells (APC), dendritic cells (DC) are specialized in capturing and processing antigens into peptide fragments that bind to MHC molecules.[1] DC are the most potent stimulators of T-cell responses and they are unique in that they stimulate not only sensitized but also naive T-cells. Thus, DC appear critical (*nature's adjuvant*) for the induction of T-cell-mediated immune responses. Recent evidence supports the role of DC in tumor immunity. In humans, DC pulsed with tumor-Id caused T-cell and humoral tumor-specific immune responses and regression of chemotherapy-resistant malignant lymphoma.[2,3] Preliminary data on DC vaccination of MM patients indicate the possibility of stimulating, in some cases, an anti-Id T-cell response. However, the immune response was not associated with any clinical benefit perhaps due to the high tumor burden of patients enrolled in the clinical trial and the use of circulating DC which appear to be less immunogenic than *ex-vivo* generated DC (*see below*).[4] DC can be generated ex-vivo from $CD34^+$ progenitor cells[1, 5,6] and more mature precursors in presence of appropriate cytokines. We recently showed that peripheral blood (PB) $CD14^+$ monocytes (Mo) from MM patients can be induced to differentiate into fully functional, mature, $CD83^+$ DC.[7] Following immunomagnetic adsorption of $CD14^+$ cells, the Mo were stimulated in serum-repleted cultures by a combination of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), with sequential use of tumor necrosis factor-α (TNF-α) as a maturation factor. The resulting $CD83^+$ DC-enriched cell population was highly efficient in priming allogeneic and autologous T lymphocytes in response to the patient-specific tumor Id. Whereas Mo-DC are efficient in tumor-Ag presentation, we have also shown that circulating DC isolated from MM patients have an impaired capacity of presenting the patient-specific Id to T cells,[8] and $CD34^+$ cells, another tested source of DC,[9] have a limited proliferative potential and provide a lower yield.[8] To evaluate the safety, the immunological and clinical responses to anti-idiotype vaccination with dendritic cells (DCs) in multiple myeloma (MM) patients, 15 patients who had failed maintenance therapy after tandem autologous stem cell transplantation were enrolled in a phase I-II clinical trial of anti-MM vaccination based on Id-pulsed DCs. DCs were differentiated from circulating $CD14^+$ monocytes in the presence of granulocyte-macrophage colony-stimulating factor and interleukin-4 and matured with a cocktail of inflammatory cytokines. Before maturation, DCs were pulsed with the autologous idiotype whole protein (=6) or idiotype (VDJ)-derived class I-restricted peptides (=9) and keyhole limpet hemocyanin (KLH). Each MM patient received a series of by-monthly immunizations consisting of three subcutaneous and two intravenous injections of increasing DC doses. DC therapy was well tolerated without clinically significant side effect. The majority of evaluable patients developed both humoral and T-cell responses to KLH suggesting immunocompetence at the time of vaccination. Seven of 14 evaluable patients developed an idiotype-specific T-cell proliferative response, 7/14 increased IFN-γ-secreting T cells and 4/14 showed an idiotype-positive delayed-type hypersensitivity test. Anti-idiotype CTL-p increased after DC vaccination in 2/2 evaluable patients. A more robust T-cell response was observed after subcutaneous DC injections and increased idiotype-specific T-cell proliferation was found up to one year after vaccination. Idiotype-derived peptides were equally effective as the whole protein in stimulating T-cell responses. Clinically, 6/14 evaluable patients have stable disease after a median of 26 months, one patient is in partial remission after 40 months, and seven patients progressed. In conclusion, injections of cryopreserved idiotype-pulsed DCs are safe and induce anti-MM immunological responses.

References

1. Bancherau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392:245-52.
2. EG Levy R. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nature Medicine 1996; 2:52-8.
3. Timmerman JM, Czerwinski DK, Davis TA, Hsu FJ, Benike C, Hao ZM, et al. Idiotype pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. Blood 2002;99:1517-26.
4. Reichardt VL, Okada CY, Liso A, Benike CJ, Stockerl-Goldstein KE, Engleman EG, et al. Idiotype vaccination using dendritic cells after autologous peripheral blood stem cell transplantation for multiple myeloma-A feasibility study. Blood 1999;93:2411-9.
5. Ratta M, Rondelli D, Fortuna A, Curti A, Fogli M, Fagnoni F, et al. Generation and functional characterization of human dendritic cells derived from $CD34^+$ cells mobilized into peripheral blood: comparison with bone marrow $CD34^+$ cells. British Journal of Haematology 1998;101: 756-65.
6. Curti A, Fogli M, Ratta M, Tura S, Lemoli RM. Stem cell factor and FLT3-Ligand are strictly required to sustain the long-term expansion of primitive $CD34^+DR^-$ dendritic cell precursors. Journal of Immunology 2001;166: 848-54.
7. Ratta M, Curti A, Fogli M, Pantucci M, Viscomi G, Tazzari PL, et al. Efficient presentation of tumor idiotype to autologous T-cells by $CD83^+$ dendritic cells derived from highly purified circulating $CD14^+$ monocytes in multiple myeloma patients. Experimental Hematology 2000;28:931-40.
8. Ratta M, Fagnoni F, Curti A, Vescovini R, Sansoni P, Oliviero B, et al. Dendritic cells are functionally defective in multiple myeloma: The role of interleukin-6. Blood 2002;100:230-7.
9. Titzer S, Christensen O, Manzke O, Tesch H, Wolf J, Emmerich B, et al. Vaccination of multiple myeloma patients with idiotype-pulsed dendritic cells: immunological and clinical aspects. British Journal of Haematology 2000;108:805-16.

CELL THERAPY OF VIRUS-RELATED NEOPLASTIC DISORDERS

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The outcome of any viral infection reflects a competition between virus replication and the capacity of the host to mount an effective immune response. In the case of viruses with rapid replication kinetics, or immune evasion tactics, persistent infection may ensue. All human viruses having oncogenic potential, such as Epstein-Barr virus (EBV), human papilloma virus (HPV), hepatitis B virus, and hepatitis C virus, establish persistent infection in the host, and continuous immunosurveillance for infected and neoplastic cells is required to prevent outgrowth of virus-induced tumors. Initial investigation in the animal model, and, more recently, the first trials in humans indicated that adoptive transfer of relatively small numbers of antigen-specific T cells could restore protective immunity and control established viral infection and virus-related disease, including cancer, in the immunosuppressed host. In particular, control of posttransplant lymphoproliferative disease by infusion of T cells specific for EBV latent antigens has been the first example of successful treatment of a virus-related neoplasia with cell therapy. Primary EBV infection occurs through a oropharyngeal route, and, in healthy individuals, is usually a self-limiting process. Infection of B cells circulating through the oropharynx results in a latent infection, with expression of only nine viral proteins (EBV nuclear antigens EBNAs -1, -2, -3A, -3B, -3C, -LP; latent membrane proteins LMPs –1, 2A, -2B) and two non-translated, EBV-encoded RNAs (EBER, BARF0). This kind of latency (latency type 3) causes immortalization of B cells in vitro, and is highly immunogenic: the emergence of virus-specific and non-specific T cell populations controls the outgrowth of EBV-transformed B-cells in vivo. However, by limiting viral gene expression (latency type 0: expression of LMP-2) and thus escaping T-cell recognition, EBV establishes a permanent latency in resting, memory B lymphocytes. Loss of host immune control, due to profound and prolonged immunosuppression occurring after transplantation, causes increased virus reactivation and an increase of virus-transformed B cells, and may lead to the development of post-transplant-associated lymphoproliferative disease (PTLD).[1] It was observed that PTLD tumor cells carried the same full array of EBV latency antigens expressed by EBV-immortalized lymphoblastoid

cell lines obtained *in vitro* by infection of resting B cell with the B95.8 EBV strain (EBV-LCL). The finding that EBV-CTLs reactivated *in vitro* from patients with mononucleosis could recognize and kill EBV-LCL led to the hypothesis that PTLD cells could also be susceptible to lysis by EBV-specific CTLs generated *in vitro* through stimulation with autologous EBV-LCL.[2] Since then, there has been ample evidence that an adoptive immunotherapy approach with transfer of lymphocytes or EBV-specific CTLs of donor origin is effective as prophylaxis and/or treatment of EBV-related PTLD in recipients of T-cell depleted hematopoietic stem cell transplantation (HSCT).[2,3] Likewise, we and others have demonstrated that infusion of autologous EBV-specific CTLs augments virus-specific immune responses, reduces viral load, and may treat overt PTLD in organ transplant (SOT) recipients.[2,4] Based on the encouraging results obtained by cellular immunotherapy with EBV-specific CTL in the prevention and treatment of the EBV-related latency III disease PTLD, pilot trials of cellular immunotherapy with polyclonal EBV CTL for other EBV-associated cancers, such as Hodgkin's disease (HD) and nasopharyngeal carcinoma (NPC), have been attempted. These pioneering work has shown the potential efficacy of T cell therapy in this setting.[5-7] However, adoptive transfer of polyclonal CTLs specific for viral latency antigens in the context of other EBV-associated malignancies is limited by the latency phenotypes displayed by tumor cells. Indeed, the immunodominant EBV-encoded antigens belong to the EBNA3 family, while CTL precursors to the EBV latency II antigens LMP1 and 2, expressed by HD and NPC, are found with low or undetectable frequency. Therefore, crucial to a strategy of T cell therapy for HD or NPC is the possibility to obtain ex vivo preferential activation and expansion of EBV CTLs specific for LMP2, the best available target antigen for immunotherapeutic control of NPC. Since CTLs reactivated by means of stimulation with EBV-LCL have specificity for the immunodominant antigens EBNA3a, b, and c, some alternative protocol for enrichment of CTLs specific for the LMP proteins is warranted. Efforts aimed at expanding the subdominant component of EBV-specific immune response directed towards latent membrane protein LMP2, by stimulation with dendritic cells genetically modified to express the antigen or pulsed with apoptotic/necrotic LCL, have been described.[8-9] Recently, it has been reported that immunization with LMP2 peptide-pulsed autologous DCs boosted epitope-specific T cell responses in NPC patients, inducing partial clinical responses in selected patients.[10] Obstacles, such as limited knowledge of immunodominant antigens, the need to enrich in specific T cell subsets, and the practical difficulty of running a cell therapy program, still limit the wide application of this strategy for treatment of virus-related tumors.

References

1. Cohen JI. Epstein-Barr virus infection. N Engl J Med 2000; 343:481.
2. Comoli P, Rooney CM. Treatment of Epstein-Barr virus infections: chemotherapy, antiviral therapy, and immunotherapy. In: Tselis A, and Jensen HB, eds. Epstein-Barr virus. New York: Taylor and Francis, 2006:353-374.
3. Rooney CM, Smith CA, Ng CY, Loftin S, Li C, Krance RA, Brenner MK. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. Lancet 1995; 345: 9.
4. Comoli P, Labirio M, Basso S, et al. Infusion of autologous EBV-specific cytotoxic T cells for prevention of EBV-related lymphoproliferative disorder in solid organ transplant recipients with evidence of active virus replication. Blood 2002; 99: 2592.
5. Bollard CM, Aguilar L, Straathof KCM, et al. Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. J Exp Med 2004; 200:1623-1633.
6. Straathof KCM, Bollard CM, Popat U, et al. Treatment of nasopharyngeal carcinoma with Epstein-Barr Virus-specific T lymphocytes. Blood 2005; 05:1898-1904.
7. Comoli P, Pedrazzoli P, Maccario R, et al. Cell therapy of stage IV nasopharyngeal carcinoma with autologous Epstein-Barr virus-targeted cytotoxic T lymphocytes. J Clin Oncol 2005; 23: 8942-8949.
8. Gahn B, Siller-Lopez F, Pirooz AD, et al. Adenoviral gene transfer into dendritic cells efficiently amplifies the immune response to the LMP2A-antigen: a potential treatment strategy for Epstein-Barr virus-positive Hodgkin's lymphoma. Int J Cancer. 2001; 93:706.
9. Subklewe M, Paludan C, Tsang ML, et al. Dendritic cells cross-present latency gene products from Epstein-Barr virus-transformed B-cells and expand tumor-reactive CD8+ killer T cells. J Exp Med 2001; 193:405.
10. Lin C, Lo W, Lee T et al. Immunization with Epstein-Barr virus (EBV) peptide-pulsed dendritic cells induces functional CD8+ T-cell immunity and may lead to tumor regression in patients with EBV-positive nasopharyngeal carcinoma. Cancer Res 2002; 62: 6952.

BCR-ABL DERIVED PEPTIDE VACCINES FOR CHRONIC MYELOID LEUKAEMIA

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In these recent years, treatment of Chronic Myelogenous Leukemia (CML) has been notably improved by imatinib mesylate a potent tyrosine kinase inhibitor that blocks the kinase activity of p210, thus inhibiting the proliferation of most CML cells. In chronic phase patients treated with imatinib, a complete cytogenetic remission (CCgR) is observed within 6-12 months of therapy in more than 80% of patients.[1] However, even in the best responders, molecular remissions are rare[2] CML stem cells appear to survive imatinib treatment[3] and recurrence of disease after drug discontinuation always occurs.[4] An alternative attempt to target CML is an active specific immunotherapy and a CML-specific vaccine approach could be considered as part of treatment's strategy in CML with the intent to eliminate and/or control residual cells by inducing a leukemia-specific immune surveillance. As a matter of fact, the chimeric p210 fusion protein resulting from the bcr-abl fusion gene produced by the t(9;22)(q34;q11) translocation (Philadelphia chromosome Ph), in virtue of the unique sequence of amino acid contained in the two alternative junctional regions (b3a2 or b2a2 fusion), can be considered a CML specific antigen potentially immunogenic.[5] In fact, within the p210 b3a2 breakpoint sequence, 5 junctional peptides were found able to bind to certain HLA class I and class II molecules and to elicit in vitro a specific T cell response both in normal donor[5] and in CML patients.[6] Subsequently p210 b3a2 breakpoint peptides were shown to induce cytotoxic T cells (CTLs) and CD4+ cells able to mediate killing and proliferation against leukemia cells[7] and finally their capability to be *endogenously* presented into class I and class II molecules of CML blasts and CML dendritic cells (DCs) have been confirmed.[8,9] All these findings furnished powerful scientific support for a b3a2-breakpoint peptides vaccine approach. The first phase I/II vaccine trials employing a mixture of 5 or more b3a2-derived peptides plus the immunological adjuvant QS-21 including non HLA restricted CML patients in chronic phase during conventional treatment showed some peptide-specific immune response but no clear disease response mediated by the vaccine.[10]

As it is more likely that effective vaccination strategies will target patients with minimal residual disease (MRD), a similar phase-II b3a2-derived peptide vaccine multicenter trial was conducted at the Department of Hematology in Siena and included patients with b3a2-CML, proper HLA restriction and established major cytogenetic response or CCgR during conventional treatment.[11] The vaccine (CMLVAX100) consisted of 5 b3a2 breakpoint-derived peptides plus QS-21 and low doses of GM-CSF as co-immunoadjuvant were also included. Sixteen CML patients showing at least 6 months established cytogenetic or molecular residual disease during prolonged treatment with imatinib or Interferon-alpha (IFN-α), were vaccinated. After 6 planned vaccinations, of 10 patients on imatinib, all improved responses with 5/10 reaching CCgR and 3/5 reaching molecular negativity. Of 6 patients on IFN-α, all but one further reduced residual disease with 2 reaching CCgR. The clinical response was associated also to a peptide-specific immune response induced by the vaccine.[13] At the present time a total of 25 b3a2-CML patients with various degrees of cytogenetically and/or molecularly defined MRD persisting after a median time of 2 years of imatinib treatment have been entered this trial. The updated results of 23/25 evaluable patients are summarized in Table 1. A further reduction of MRD, including some complete molecular remissions (CMR), after vaccinations has been observed in about 65% of this extended series of patients . In conclusion, vaccination with bcr-abl derived b3a2 peptides was followed by reduction of long maintained residual disease in most of patients and by achievement of CMR in some of them and we are currently evaluating a peptide vaccine strategy also for patients with b2a2-CML. However, phase III randomized trials comparing imatinib alone vs imatinib plus CML-specific peptide vaccines are necessary to assess the role of this additive immune-mediated anti-leukemic effect for eradication or long-term control of residual disease.

Table 1. Disease response after vaccinations with CMLVAX100 plus QS-21 and GM-CSF: updated results of the phase II clinical trial. A enrollment, the median time of imatinib treatment and the median time of established residual disease were 24 months (range 12-50) and 12 months (range 6-33), respectively.

Disease status at enrolment (time 0)(3 months)	*Response after 6 CMLVAX100 (18 months)*		*Response after 3 additional boosts (32 months)*		*Response after 6 additional boosts*	
	23 patients		11 patients		6 patients	
Major/minor Cytogenetic Response	10	6 CCγR* (4 CMR**) 2 improved 2 stable disease	8	5 CCγR 2 maintaining CMR 1 improved	6	1 CCγR 1 >1 log improved 3 CMR 1 relapse (never CMR)
Complete Cytogenetic Response	10	7 > 1 log improved (2 CMR) 3 stable disease	3	1 improved (CMR) 1 maintaining CMR 1 CCgR	/	/
Hematologic Response	3	3 no response	/		/	/

References

1. O'Brien SG, Guilhot F, Larson LA et al. Imatinib compared with interferon and low dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukaemia. N Engl. J Med 2003, 348 :994-1004.
2. Cortes J, Talpaz M, O'Brien S et al . Molecular responses in patients with Chronic Myelogenous Leukemia in Chronic Phase Treated with Imatinib Mesylate. Clin Cancer Res 2005, 11:3425-3432.
3. Cortes J, O'Brien S, and Kantarjian H. Discontinuation of imatinib therapy after achieving a molecular response. Blood 2004, 104 : 2204-2205.
4. Graham SM, Jorgensen HG, Allan E et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood 2002, 99:319-25.
5. Bocchia M, Korontsvit T, Xu Q, et al. Specific human cellular immunity to BCR-ABL oncogene-derived peptides. Blood 1996, 87:3587-92.
6. Bosh GJ, Joosten AM, Kessler JH, Melief CJ, Leeksma OC. Recognition of BCR-ABL positive leukemia blast by human DC4+ T cells elicited by primary in vitro immunization with a BCR-ABL breakpoint peptide. Blood 1996;88:3522-7.
7. Mannering SI, McKenzie JL, Fearnley DB, Hart DN. HLA-DR1-restricted bcr-abl (b3a2-specific CD4+ T lymphocytes respond to dendritic cells pulsed with b3a2 peptide and antigen-presenting cells exposed to b3a2 containing cell lysates. Blood 1997; 90:290-7.
8. Clark RE, Dodi AI, Hill SC, et al. Direct evidence that leukemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein. Blood 2001;98:2887-93.
9. Yasukama M, Ohminami H, Kojima K, et al. HLA class II-restricted antigen presentation of endogenous bcr-abl fusion protein by chronic myelogenous leukemia-derived dendritic cells to CD4+ T lymphocytes. Blood. 2001, 98:1498-1505.
10. Cathcart K, Pinilla-Ibarz J, Korontsvit T et al. A multivalent bcr-abl fusion peptide vaccination trial in patients with chronic myeloid leukemia. Blood 2003, 103:1037-42.
11. Bocchia M, Gentili S, Abruzzese E et al. Effect of a p210 multipeptide vaccine associated with Imatinib or Interferon in patients with chronic myeloid leukemia and persistent residual disease: a multicentre observational trial. Lancet 2005, 365: 657-62.

NATURAL KILLER CELL ALLOREACTIVITY TO ERADICATE LEUKEMIA AND REDUCE TRM AFTER HAPLOIDENTICAL HEMATOPOIETIC TRANSPLANTATION

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Full HLA haplotype-mismatched (haploidentical) transplants are necessary because many leukemia patients, who might benefit from allogeneic hematopoietic transplantation, still fail to find a matched graft whether related or unrelated. In mismatched transplants T cell alloreactions are totally unmanageable because of the high frequency of alloreactive donor T cells recognizing the host major antigens and would cause lethal GvHD. Only extensively T cell-depleted stem cell transplants engraft across the HLA barrier without GVHD.[1-3] T cell depletion is underlies for the major reasons for transplant failure: leukemia relapse, infection and slow recovery of functional T cell immunity to pathogens is responsible for 35% infection-related mortality.

In an attempt to develop new strategies to protect patients from leukaemia relapse and infections, while not causing GvHD, we concentrated on Natural Killer (NK) cells. Although NK cells are primed to kill by signals delivered through several, different activating receptors, they do not kill autologous because they also express inhibitory receptors that recognize self-MHC class I molecules. Some of these inhibitory receptors are known as killer cell Ig-like receptors (KIR) and are specific for epitopes shared by HLA class I allele groups ("KIR ligands"). As KIRs are clonally distributed, the NK cell population in any given individual will be constituted of a repertoire of NK cells with different allospecificities. Some NK cells mediate alloreactions when the mismatched allogeneic target cells do not express the class I alleles that block them. When faced with mismatched allogeneic targets, these NK clones sense the missing expression of self HLA class I alleles and mediate alloreactions.[4-8] KIR ligand mismatches in the GvH direction trigger donor vs recipient NK cell alloreactions which improve engraftment, do not cause GvHD and control relapse in acute myeloid leukemia patients.[6-10]

The mechanism whereby alloreactive NK cells exert their benefits in transplantation has been elucidated in mouse models. In F1 H-2d/b♀parent H-2b transplants, donor NK cells that do not express the H-2b-specific Ly49C/I inhibitory receptor (and bear H-2d-specific Ly49A/G2 receptors), are activated to kill the recipient's targets. In these murine MHC haploidentical bone marrow transplant models, infusion of alloreactive NK cells 1) ablates leukemic cells, 2) ablates recipient T cells which reject the graft, and 3) ablates recipient dendritic cells (DCs) which trigger GvHD, thus protecting from GvHD.[10]

Unexpectedly, in these mouse models we now find that NK cell alloreactivity also boosts very rapid rebuilding of donor adaptive immunity to infections. Conditioning with TBI plus the infusion of alloreactive NK cells promotes an extremely accelerated recovery of donor-type B and T cell precursors and dendritic cells (DCs). Very quickly, B and T cell precursors expand, mature correctly (e.g., T cell repertoires include regulatory cells) and home to peripheral lymphoid tissues where, in concert with the rapidly reconstistuting DCs, they protect mice from infectious challenges. What about the echanism(s)♀ We find that 1) specific interaction between donor alloreactive NK cells and recipient-type DCs is required for accelerated immune rebuilding; 2) NK:DC interaction results in stable conditioning of bone marrow and thymus microenvironements. In fact, NK conditioned mice remain receptive to accelerated immune rebuilding even when transplanted many days after NK conditioning; 3) quantitative PCR on bone marrow and thimus of NK conditioned mice shows several-fold increased expression of cytokines implicated in B and T cell maturation, such as, IL-7 and c-Kit ligand; 4) Finally and most interestingly, the accelerated immune rebuilding effect can be reproduced by conditioning mice with the infusion of short-term (3-hour) NK:DC co-culture supernatants (Ruggeri et al., manuscript in preparation).

These observations have prompted an analysis of infectious mortality in the entire series of 178 acute leukemia patients (AML and ALL) who received haploidentical transplant at our Centre. Strikingly, this analysis has unravelled that transplantation from KIR ligand-mismatched (i.e., NK alloreactive) donors, in addition to controlling AML relapse, offers statistically significant protection from infectious mortality in AML and in ALL patients. Moreover, by multivariate analysis, its effect appears to be the only significant predictor of lower incidence of infectious mortality, when compared with diagnosis (ALL vs AML) or disease status at transplant (remission vs relapse). Altogether, these observations indicate that yet to be identified "immune rebuilding factor(s)", produced in consequence of the interaction between donor alloreactive NK and recipient-type DCs, might be exploited to boost recovery of adaptive immunity to infections and help reduce infection mortality after haploidentical hematopoietic transplantation.

References

1. Aversa F, Tabilio A, Terenzi A, et al. Successful engraftment of T-cell-depleted haploidentical "three-loci" incompatible transplants in leukemia patients by addition of recombinant human granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells to bone marrow inoculum. Blood. 1994;84:3948-3955.
2. Aversa F, Tabilio A, Velardi A, et al. Treatment of high-risk acute leukemia with T-cell-depleted stem cells from related donors with one fully mismatched HLA haplotype. N Engl J Med. 1998;339:1186-1193.

3. Aversa F, Terenzi A, Tabilio A, et al. Full haplotype-mismatched hematopoietic stem-cell transplantation: a phase II study in patients with acute leukemia at high risk of relapse. J Clin Oncol. 2005;23:3447-3454.
4. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today. 1990;11:237-244.
5. Karre K. A perfect mismatch. Science. 2002;295:2029-2031.
6. Velardi A, Ruggeri L, Moretta A, Moretta L. NK cells: a lesson from mismatched hematopoietic transplantation. Trends Immunol. 2002;23:438-444.
7. Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. Blood. 2002;100:1935-1947.
8. Ruggeri L, Mancusi A, Capanni M, Martelli MF, Velardi A. Exploitation of alloreactive NK cells in adoptive immunotherapy of cancer. Curr Opin Immunol. 2005;17:211-217.
9. Ruggeri L, Capanni M, Casucci M, et al. Role of natural killer cell alloreactivity in HLA-mismatched hematopoietic stem cell transplantation. Blood. 1999;94:333-339.
10. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295:2097-2100.

A PHASE I STUDY OF IMMUNIZATION OF INDOLENT NON HODGKINS LYMPHOMA PATIENTS WITH AUTOLOGOUS MONOCYTE-DERIVED DENDRITIC CELLS LOADED WITH HEAT SHOCKED AND KILLED AUTOLOGOUS TUMOR CELLS

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B-cell malignancies result from the clonal expansion of a single B cell at distinct stages of differentiation. Currently available therapeutic options for indolent B-cell lymphoma, such as standard chemotherapy, high-dose chemotherapy, and bone marrow transplantation, have improved the outcome of these diseases.[1] However, the majority of patients are not cured and other approaches, including immunotherapy, are being currently investigated, based on the evidence indicating that these tumors may be responsive to T-cell–mediated immunity in vivo. Although immunization with tumor-derived idiotype protein is a frequently used procedure[2,3,4] vaccination with DCs loaded with killed tumor cells may activate response to a much wider range of antigens, without requiring prior molecular identification of such determinants.[5] Furthermore, such DC-based vaccines could be available to all patients, irrespective of the HLA type. To evaluate the safety and tolerability of this approach, 18 patients with measurable relapse/refractory follicular lymphoma (FCL; n= 12), lymphoplasmocytoid lymphoma (n= 5) and chronic lymphatic leukemia (CLL; n=1) have been enrolled in a phase I study. Median prior number of treatment regimens was 2 (range 1-5) comprising 4 patients treated with high-dose chemotherapy supported by autologous stem cell transplantation. The vaccination was started after at least 6-months from the last chemotherapy treatment. All patients were evaluable for toxicity and 16/18 patients for efficacy with a median follow-up of 16.5 months (range 6-33 months). Each patient received 4 intradermal/subcutaneous injections at 2-weekly intervals of 50×10^6 tumor-loaded DCs. Immature DCs were generated by 5-days culture of autologous monocytes in the presence of IL-4 and GM-CSF.[6] After selection by immunomagnetic technique, autologous $CD19^+$ tumor cells, harvested from lymph nodes (n= 12) and/or peripheral blood (n= 6), were heat shocked and then irradiated by UVC.[7] DCs were loaded for 48 hrs with killed tumor cells and then, to induce their maturation, were cultured for 12 hrs in the presence of TNF-α. Overall, vaccinations were well tolerated and no autoimmune reactions were observed. Mild erythema in the site of injection developed in the majority of patients (12/18), but only in 2 cases induration and extended erythema was observed. Six of 16 (37.5%) evaluable patients had objective responses. Two patients had partial responses (PR). One is still in PR and the other had a PR lasting 7 months. Four patients had complete remission (CR). Two patients are still in CR and the other 2 patients had a mean CR duration of 14.5 months. The remaining 10 patients had stable disease (n=5) or progressive disease (n=5). The overall monitoring of immune responses is ongoing. However, in one patient in PR, we evaluated the frequency of anti autologous tumor-specific T cells, by ELISPOT assay for IFN-gamma, on pathologic lymph nodes harvested before and after 2 months from the last vaccination. A significant increase of specific T-cell frequency was observed in the post-vaccination lymph node, compared to the tissue sample taken before vaccination. Moreover, evaluation of CD8+ T cell maturation markers, by analysis for CCR7 and CD45RA expression, indicated a shift of tumor-infiltrating T cells towards memory and effector stages in the lymph-node isolated after vaccination. In conclusion, injection of DCs loaded with killed tumor cells is a well-tolerated procedure achieving clinical and immunological responses also in the presence of significant tumor burden. However, further strategies, following DC-vaccination, are needed to ensure durable immune and clinical responses.

References

1. Hennessy BT, Hanrahan EO, Daly PA. Non-Hodgkin lymphoma: an update. Lancet Oncol 2004;5:341-53.
2. Kwak LW, Campbell MJ, Czerwinski DK, Hart S, Miller RA, Levy R.: Induction of immune responses in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumors. N Engl J Med 1992;327:1209-15.
3. Timmerman JM, Czerwinski DK, Davis TA, et al. Idiotype-pulsed dendritic cell vaccination for B cell lymphoma: clinical and immune responses in 35 patients. Blood 2002;99:1517-26.
4. Weng WK, Czerwinski D, Timmerman J, Hsu FJ, Levy R. Clinical outcome of lymphoma patients after idiotype vaccination is correlated with humoral immune response and immunoglobulin G Fc receptor genotype. J Clin Oncol 2004;22: 4717-24.
5. Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature;392:86-89, 1998
6. Sallusto F, Lanzavecchia A Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor-a. J Exp Med 1994;179:1109-18.
7. Di Nicola M, Napoli S, Anichini A, et al. Dendritic cell viability is decreased after phagocytosis of apoptotic tumor cells induced by staurosporine or vaccinia virus infection. Haematologica 2003; 88:1396-404.

BEST ABSTRACTS

BEST-01

THE APOPTOTIC RESPONSE OF NUCLEAR BCR-ABL REQUIRES P73

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The BCR-ABL oncoprotein of Chronic Myelogenous Leukemia (CML) localizes to the cell cytoplasm where activates different proliferative and anti-apoptotic signaling pathways. We have previously reported that when BCR-ABL active kinase is displaced from its cytoplasmatic localization and forced inside the nucleus it can cause cell death. To identify nuclear proteins involved in the apoptotic response triggered by the nuclear entrapment of the BCR-ABL oncoprotein, we engineered a BCR-ABL-Nuk construct that mostly localizes to the nuclear compartment. We found that this nuclear BCR-ABL interacts with p73 through its SH3 domain and phosphorylates p73 on tyrosines 99 and 121. The stabilization and tyrosine phosphorylation of the p73 induces cell death. A kinase defective BCR-ABL-Kd-Nuk is unable to elicit such a response. Death induced by BCR-ABL-Nuk can be suppressed by the co-expression of a p73 mutant bearing two phenylalanines in place of tyrosine 99 and 121. In addiction, by transient expression of BCR-ABL-Nuk in p73-null Mouse Embryo Fibroblasts (MEFs), we further confirmed that nuclear BCR-ABL requires p73 to induce apoptosis. In fact, in this cellular background, expression of a nuclear BCR-ABL did not cause an apoptotic response. However, reconstitution of knock-out MEFs with p73 promptly restored BCR-ABL-Nuk-induced cell death. Our data identify p73 as a nuclear target of BCR-ABL-Nuk and suggests that the interaction between these two proteins is responsible for the apoptotic response caused by nuclear BCR-ABL.

BEST-02

DIAGNOSTIC AND PROGNOSTIC SIGNIFICANCE OF A 8 GENE SUBSET, IDENTIFIED THROUGH GENE EXPRESSION PROFILING, IN IDIOPATHIC MYELOFIBROSIS

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Idiopathic myelofibrosis (IM) is a clonal disorder of the hematopoietic multipotent stem cell, that is included with Polycythemia Vera (PV), Essential Thrombocythemia (ET) and other less frequent entities among the Ph'-neg myeloproliferative diseases (MPD). Despite recent advances in the definition of diagnostic and prognostic criteria in patients with IM and the characterization of some cellular abnormalities, the molecular defect(s) associated with the development of this disorder remains unknown, notwithstanding the recent description of the Val617Phe mutation in the exon12 of JAK2 gene that can be found in about half of patients. We have used a transcriptome comparative microarray analysis to address aberrantly regulated genes in IM; the gene expression profile of IM CD34+ cells, obtained from the peripheral blood, was compared to that of normal, bone marrow-derived, cells. For this purpose, we prepared three pools of purified (purity >98%) CD34+ cells from IM subjects, and two pools from normal donors, each comprising five subjects. The cDNA was hybridized to an Affymetrix HG-U133A oligonucleotide microarray chip representing 22,283 probesets. Two-hundred eighteen differentially expressed genes were identified and 47 among these, that we considered as potentially important in the pathophysiology of IM, were validated by quantitative PCR, with an overall validation rate of 77%. According to a class prediction analysis, we identified a set of eight gene markers (CD9, GAS2, DLK1, CDH1, WT1, NFE2, HMGA2 and CXCR4) that would allow to recognize normal from IM CD34+ cells in 100% of examined cases. These gene were found to be aberrantly regulated also in the granulocytes of IM, PV and ET patients; class prediction analysis differentiated IM from normal granulocytes in 100% of cases, while a correct disease class attribution was obtained in 95% of IM, PV, or ET patients. The abnormal expression of HMGA2 and CXCR4 was found to associate with the presence of JAK2V617F mutation, while that of CD9, DLK1,GAS2, CDH1, NFE2 and WT1 was not affected by the presence of the mutation. Altered gene expression was also corroborated by the detection of abnormally high CD9 or CD164, and low CXCR4, protein content in CD34+ cells, that differentiated IM from normal, PV or ET patients. Interestingly, CXCR4 is significantly down-regulated on IM CD34+ cells and we speculate that it might be related to the constitutive mobilization of CD34+ cells in these patients. We also found that CD9 and DLK1 expression levels were directly ($r=0.56$, $p<001$) and inversely ($r= -0.58$, $p<0.001$) related, respectively, to the platelet count. Higher WT1 expression levels identified IM patients with more active disease, as evidenced by elevated CD34+ cell count and higher severity score. In summary, we have demonstrated the possibility to use a defined set of molecular markers, such as the eight genes described herein, to approach the diagnosis and resolving the phenotypic overlapping among the different clinical forms of MPD. Furthermore, determination of WT1 expression levels in IMF patients might be suitably employed as the first molecular marker of disease activity and, prospectively, prove useful also to measure response to therapy.

BEST-03

FUNCTIONAL CHARACTERIZATION OF CYTOMEGALOVIRUS (CMV)-SPECIFIC CD4 AND CD8 T CELL LINES GENERATED BY USING PROTEIN-SPANNING POOLS OF PP65 AND IE1 DERIVED PEPTIDES

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Background and Aims. Reactivation of latent CMV in immunocompromised recipients of allogeneic stem cell transplantation remains a major cause of morbidity and mortality. Reconstitution of immunity by CMV-specific immunotherapy is an attractive alternative to standard treatment. In this study, we have analyzed the functional properties of CMV-specific T cells generated by using protein-spanning pools of CMV-derived peptides. *Methods.* Dendritic Cells (DC) were differentiated from donor peripheral blood monocytes after a 7-day culture in the presence of GM-CSF plus IL-4 and matured with TNFα, IFNα, IFNγ, IL1β, POLI I:C. Matured DC were then pulsed with a pool of 48 peptides spanning pp65, IE1, pp150 and pp50 proteins, which are recognised by both CD4 and CD8 T lymphocytes. Donor T cells were stimulated weekly three times at a T cell/DC ratio of 6:1 with mature peptide pulsed-DC. At the end of the culture T cells were analyzed for their phenotypic and functional features. *Results.* CD4 and CD8 CMV-specific T cell lines were successfully expanded from 10 CMV seropositive donors. Cultured T cells expressed CD8 (mean 70%, range 60-81%) and CD4 (mean 20%, range 15-28%) and showed a Effector Memory (mean 26%, range 19-30%) or a Effector Memory RA-Positive phenothype (mean 67%, range 59-77%). An enriched CMV-specific T cell population was observed after pentamers staining (7-45% pentamer-positive T cells). In all cases, cultured T cells showed a cytolitic activity against CD8-peptide pulsed target cells (average lysis 50%, range 40-55%) and, to a lesser extent, against CD4-peptide pulsed target cells (average lysis 35%, range 30-40%). In addition, cultured T lymphocytes were able to proliferate and to produce intracytoplasmic IFN-γ;(average production 50%, range 35-60%) after exposure to peptide-pulsed DC. CMV-specific T cells were also analysed for the expression of adhesion molecules and chemokine receptors and for their ability to migrate in response to inflammatory (CXCL9, CCL3 and CCL5) and constitutive (CXCL12) chemokines. T cells showed high levels of CXCR3 (average expression 94%, range 81-99%), CCR1 (average expression 61%, range 57-92%), and to a lesser extent, CXCR4 (mean 25%, range 10-61%). In accordance with this profile, cultured T cells strongly migrated in response to CXCL12 (mean Migration Index (MI) 1.8, range 1.5-2), CCL5 (mean MI 7.2, range 5.4-11), CCL3 (mean MI 2.3, range 1.3-4.2) and CXCL9 (mean MI 2, range 1.5-2.7), which are involved in the recruitment of effector cells to peripheral sites of viral infection. Finally, CMV-specific T cells showed high level of CD49d (β98%), which guides the extravasation of effector cells into inflamed tissues, and low levels of CD62L, a molecule involved in the migration to lymphoid organs. *Conclusions.* In conclusion, we demonstrated the possibility to generate activated and armed CMV-specific T cells, poten-

tially able to reach viral-infected tissues and to recognize and kill CMV-infected cells.

BEST-04

CLINICOBIOLOGICAL FEATURES IN CHRONIC LYMPHOCYTIC LEUKEMIA WITH 14Q32/IGH TRANSLOCATIONS

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Chromosomal translocations represent a rare event in B-cell Chronic Lymphocytic Leukemia (B-CLL). Furthermore, in opposition to other chronic lymphoprolipherative diseases, translocations of 14q32 involving the IgH loci usually occur at a <5% frequency in B-CLL. We studied 551 cases of B-CLL by conventional cytogenetic analysis and by FISH using a 5 probe-panel (14q32/IgH, 11q/ATM, 17p/p53, 13q14, +12) to identify those cases bearing a 14q32 break and/or IgH gene translocation as primary abnormality in order to define the clinicobiological features associated with this genetic change. Cases with t(11;14) or BCL1 rearrangement were not included. We found 30/551 cases (5,4%) with 14q32/IgH break by interphase FISH (46-83% of interphases). The translocation partner could be so far identified in 8 patients (6 with 18q21, 1 with 2p12, 1 with 6p12). Additional abnormalities were detected in 11/30 cases in a minority of nuclei (13-70%): 7 cases with 13q-, 3 cases with +12, 1 case with 17p-. B-CLL cases with 14q32/IgH rearrangement showed atypical morphology in 16,6% of cases and classical immunophenotype (4/5 points immunophenotypic score according to Matutes *et al.*) in 50% of cases. Hypermutation of the IGV_H gene sequences were found in 42,8% of the cases tested. The 14q32/CLL cytogenetic entity was compared with 234 cases with favourable karyotype (normal or 13q-), 220 cases with intermediate risk (1-2 anomalies, +12) and 67 cases with unfavourable karyotype (complex, 11q-, 17p-). The 14q32/IgH group showed a high frequency of adenopathy and splenomegaly compared to other CLLs. Comparison of the survival curves for these groups showed that the presence of a 14q32/IgH rearrangement adversely affected outcome with respect to the favourable group (p= 0.0013) while resulting in a better outcome when tested against the unfavourable group (p= 0.0051). No significant difference was noted when the 14q32 group was compared against the intermediate group (p=0.5). These findings show that those patients with primary 14q32/IgH translocation have peculiar clinicobiological features and that they should be allocated in an intermediate risk category.

BEST-05

MOLECULAR CHARACTERIZATION OF POSTTRANSPLANT LYMPHOPROLIFERATIVE DISORDERS DERIVED FROM DONOR TRANSPLANTED LYMPHOCYTES AND OCCURRING IN LIVER TRANSPLANT PATIENTS

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Post-transplant lymphoproliferative disorders (PTLD) represent a frequent source of morbidity and mortality among solid organ transplant recipients receiving immunosuppressive therapy. Most PTLD occurring in solid organ patients arise from recipient cells, whereas only a fraction of cases derive from donor transplanted lymphocytes. Donor-derived PTLD (D-PTLD) usually have a predilection for the allograft. The factors that determine the outgrowth of donor B cells and their peculiar intragraft location are still unknown. To clarify the histogenesis and the pathogenesis of D-PTLD, we investigated a panel of 16 monoclonal PTLD occurring in liver transplant patients, including 9 cases arising from donor cells and 7 cases arising from recipient cells (R-PTLD). Several phenotypic and genotypic markers of B-cell histogenesis were analyzed. Phenotypic markers of histogenesis included expression of BCL6, MUM1 and CD138, which segregate the germinal center (GC) stage of B-cell differentiation (BCL6+/MUM1±/CD138-) from later stages of maturation (BCL-6-/MUM1+/CD138±). Genotypic markers of histogenesis were represented by somatic hypermutation of immunoglobulin variable (IGV) genes, a phenomenon experienced by B-cells during the GC reaction. To define the role of antigen in the pathogenesis of the disease, we also analyzed the usage and the mutational profile of clonal IGV heavy (IGHV) and IGV light chain gene rearrangements. Finally, D-PTLD and R-PTLD were investigated for the presence of aberrant somatic hypermutation of proto-oncogenes. Toward this aim, a region spanning up to 1.5 Kb from the transcription start site of the proto-oncogenes PAX5, RHO/TTF, PIM1 and cMYC was analyzed by PCR amplification and direct sequencing. All D-PTLD were EBV-infected lymphoproliferations morphologically classified as polymorphic PTLD (P-PTLD), whereas R-PTLD were classified as diffuse large B-cell lymphomas and EBV infection was restricted to 2 cases. Median time from transplant to PTLD was 7 months (range 5-25) for D-PTLD and 64 months (range 6-110) for R-PTLD. Analysis of phenotypic markers of B-cell histogenesis showed expression of the phenotypic profile BCL6+/MUM1-/CD138- in 4 DLBCL with centroblastic features, all arising from recipient cells. The phenotypic profile BCL6-/MUM1+/CD138±, consistent with a post-GC stage of pre-terminal B-cell differentiation, was detected in 8/14 PTLD, including 6/7 D-PTLD and 2/7 R-PTLD. A clonal IGV rearrangement was identified in all cases. Analysis of somatic hypermutation showed the presence of somatically hypermutated IGV genes in 11/16 PTLD. Unmutated IGHV rearrangements were identified in 3/9 D-PTLD and in 2/7 R-PTLD. Analysis of intraclonal heterogeneity showed the presence of ongoing mutations in 1/3 D-PTLD. In all the other cases, intraclonal heterogeneity was absent. Analysis of the distribution of individual IGHV rearrangements disclosed differences between D-PTLD, R-PTLD and normal repertoire. In particular, the IGHV4-39 gene was the most frequently rearranged IGHV gene in D-PTLD (3/7 cases); conversely, IGHV4-39 was absent in R-PTLD and relatively rare in the non-neoplastic B-cell repertoire. Concerning light chain genes, a significant overrepresentation of lambda chain variable gene rearrangements (IGLV) was observed both in D-PTLD (7/8 cases) and in R-PTLD (5/6 cases), when compared to the normal B-cell repertoire. Two R-PTLD displayed inactivated IGLV rearrangements by crippling mutations. Aberrant somatic hypermutation of proto-oncogenes was found in 5/9 D-PTLD and 2/6 R-PTLD. PAX5 was mutated in 3/9 cases, including 2 D-PTLD and 1 R-PTLD; RhoH/TTF was mutated in 1 R-PTLD; PIM-1 was mutated in 2 D-PTLD and cMYC was mutated in 3 D-PTLD and 2 R-PTLD. The mutation pattern was similar between D-PTLD and R-PTLD and was consistent with the somatic hypermutation process. In conclusion, our data suggest that both D-PTLD and R-PTLD occurring in liver transplant patients arise from a B-cell subset phenotypically mimicking post-GC, pre-terminal differentiated B-cells. Lack of IGV mutations, however, suggests that a fraction of cases failed to perform a proper GC reaction. The

biased usage of the IGHV4-39 gene and of lambda genes suggests that antigen stimulation and selection might have a role in the pathogenesis of D-PTLD. Finally, the presence of aberrant somatic hypermutation in a large fraction of D-PTLD suggests that the *infidelity* of the IG hypermutation machinery may play an important role in the genesis and progression of both R-PTLD and D-PTLD.

BEST-06

THE PERSISTANCE OF RESIDUAL VEIN THROMBOSIS, AFTER AN EPISODE OF DEEP VEIN THROMBOSIS, AND THE RISK OF NEW OVERT CANCER AND CARDIOVASCULAR DISEASE. PERSISTENZA DEL TROMBO VENOSO RESIDUO E RISCHIO DI NUOVI EPISODI DI CANCRO E/O MALATTIA CARDIOVASCOLARE

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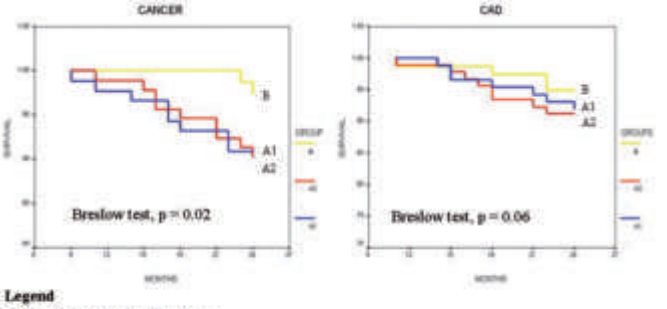
Background. We have recently demonstrated that the presence of Residual Vein Thrombosis (RVT), UltraSonography (US)-detected at the 3rd month after an episode of Deep Vein Thrombosis (DVT) of the lower limbs, is an independent risk factor for developing recurrent Venous Thromboembolism (VTE). The management of DVT patients by detection of RVT may, therefore, represent a simple and reproducible method for establishing the individual risk of recurrence and for tailoring the optimal duration of Oral Anticoagulants (OA) (Siragusa S *et al.* Blood 2003;102(11):OC183a). At the present, it is unknown whether RVT may also identify patients at increased risk for cancer and/or cardiovascular disease (CD). Objective of the study. In patients with DVT of the lower limbs, we conducted a prospective study for evaluating the correlation between RVT and the risk of new overt cancer and/or CD. *Materials and methods.* Consecutive patients, with an episode of idiopathic or provoked DVT, were evaluated after 3 months from the index DVT; presence/absence of RVT was detected and patients managed consequently (Table). The incidence of VTE recurrence, overt cancer and new CD was evaluated over a period of 3 years after the index DVT. Survival curves (Kaplan-Mayer) and related Breslow test have been used for statistics. *Results.* Three-hundred fourty-five patients were included in the analysis. The results are listed in Table and Figures below.



Legend
RVT: Residual Vein Thrombosis
CAD: Cardiovascular Disease
A1: Presence of RVT (patients received 12 months of anticoagulants from the index DVT)
A2: Presence of RVT (patients received 3 months of anticoagulants from the index DVT)
B: Absence of RVT (patients received 3 months of anticoagulants from the index DVT)

Figure. Relationship between RVT and subsequent Cancer and/or Cardiovascular Event.

Table. Incidence of events over a period of 3 years accordingly to RVT findings.

Group	*Number of patients*	*Presence of RVT at the 3rd month of OA from the index DVT*	*Duration of OA from the index DVT*	*Incidence of recurrent VTE*	*Incidence of new cancer*	*Incidence of new CD*
Group *A1	142	yes	12 months	11 (7.7%)	8 (5.6%)	7 (4.9%)
Group *A2	91	yes	3 months	16 (17.5%)	9 (9.9%)	7 (7.7%)
Group B	112	no	3 months	1 (0.9%)	3 (2.6%)	4 (3.5%)

**Part of these patients were originally randomized to receive 3 or 12 months of OA; RVT (Residual Vein Thrombosis); OA (Oral Anticoagulants); DVT (Deep Vein Thrombosis); VTE (Venous Thromboembolism); CD (Cardiovascular Disease).*

The incidence of recurrent VTE and new overt cancer was statistically lower in patients without RVT than in those with RVT; no significant differences were found in the incidence of new CD. These data are applicable in patients with idiopathic or provoked index DVT. In patients with RVT, the advantage of prolonging anticoagulation for 12 months was lost at the end of the treatment. *Conclusions.* This is the first study evaluating the relationship between US-detected RVT and the risk of developing cancer and CD; RVT presence, at 3rd month from the index DVT, is an independent risk factor for recurrent VTE and indicates patients at risk for new overt cancer. This risk remains over a period of 3 years, independently whether index DVT was idiopathic or provoked. In these patients, the advantage of indefinite anticoagulation should be assessed in properly designed study.

ORAL COMMUNICATIONS

Hematopoietic Stem Cell Transplantation

CO-001

ALLOREACTIVE NATURAL KILLER CELLS REBUILD ADAPTIVE IMMUNITY TO INFECTIONS AFTER HAPLOIDENTICAL HEMATOPOIETIC TRANSPLANTATION

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In haploidentical hematopoietic transplantation, donor-versus-recipient natural killer (NK) cell alloreactivity derives from a mismatch between inhibitory Killer Cell Ig-like Receptors (KIRs) for self HLA class I molecules on donor NK clones and their HLA class I ligands (KIR ligands) on recipient cells. When faced with mismatched allogeneic targets, these NK clones sense the missing expression of self HLA class I alleles and mediate alloreactions. KIR ligand mismatches in the GvH direction trigger donor vs recipient NK cell alloreactions which improve engraftment, do not cause GvHD and control relapse in acute myeloid leukemia patients (Ruggeri *et al.*, Science 2002; updated 2006). The mechanism whereby alloreactive NK cells exert their benefits in transplantation has been elucidated in mouse models. In F1 H-2d/b parent H-2b transplants, donor NK cells that do not express the H-2b-specific Ly49C/I inhibitory receptor (and bear H-2d-specific Ly49A/G2 receptors), are activated to kill the recipient's targets. In these murine MHC haploidentical bone marrow transplant models, infusion of alloreactive NK cells 1) ablates leukemic cells, 2) ablates recipient T cells which reject the graft, and 3) ablates recipient dendritic cells (DCs) which trigger GvHD, thus protecting from GvHD (Ruggeri *et al.*, Science 2002). Unexpectedly, in these mouse models, we now find that NK cell alloreactivity also boosts very rapid rebuilding of donor adaptive immunity to infections. Conditioning with TBI plus the infusion of alloreactive NK cells promotes an extremely accelerated recovery of donor-type B and T cell precursors and DCs. Very quickly, B and T cell precursors expand, mature correctly (e.g., T cell repertoires include regulatory cells) and home to peripheral lymphoid tissues where, in concert with the rapidly reconstistuting DCs, they protect mice from infectious challenges. What about the mechanism(s)? We find that 1) specific interaction between donor alloreactive NK cells and recipient-type DCs is necessary and sufficient for accelerated immune rebuilding; 2) NK:DC interaction results in stable conditioning of bone marrow and thymus microenvironements. In fact, NK conditioned mice remain receptive to accelerated immune rebuilding even when transplanted many days after NK conditioning; 3) quantitative PCR on bone marrow and thimus of NK conditioned mice has not shown increased expression of any of the cytokines known to be produced by NK cells and/or DCs; 4) finally and most interestingly, the accelerated immune rebuilding effect can be reproduced by conditioning mice with the infusion of short-term (3-hour) NK:DC co-culture supernatants. While proteomics approaches are been applied to identify the factor(s) putatively responsible for the immune rebuilding effect, these observations have prompted an analysis of infectious mortality in the entire series of 178 acute leukemia patients (AML and ALL) who received haploidentical transplant at our Center from 1993 through 2006. Strikingly, this analysis has unravelled that transplantation from KIR ligand-mismatched (i.e., NK alloreactive) donors, in addition to controlling AML relapse, offers statistically significant protection from infectious mortality in AML and also in ALL patients. Moreover, by multivariate analysis, NK alloreactivity appears to be the only significant predictor of lower incidence of infectious mortality, even when compared with diagnosis (ALL vs AML) or disease status at transplant (remission vs relapse). Altogether, these observations indicate that yet to be identified *immune rebuilding factor(s)*, produced in consequence of the interaction between alloreactive NK and recipient-type DCs, might be exploited to boost recovery of adaptive immunity to infections and help reduce infection mortality after haploidentical hematopoietic transplantation.

CO-002

INCIDENCE OF SECONDARY MYELODYSPLASTIC SYNDROME/ACUTE LEUKEMIA IN 1088 LYMPHOMA PATIENTS FOLLOWING HIGH-DOSE THERAPY AND AUTOGRAFT: A STUDY FROM GITIL (GRUPPO ITALIANO TERAPIE INNOVATIVE NEI LINFOMI)

Zanni M, Magni M, Rambaldi A, Rosato R, Patti C, Ciceri F, Gallamini A, Cortelazzo S, Majolino I, Benedetti F, Corradini P, Boccadoro M, Barbui T, Gianni AM,Tarella C

on behalf of GITIL (Gruppo Italiano Terapie Innovative Nei Linfomi)

Introduction. High-dose therapy with autologous stem cell transplantation is an effective treatment option for both non-Hodgkin s Lymphoma (NHL) and Hodgkin s Lymphoma (HL). Its clinical applicability has been considerably amplified by using peripheral blood progenitor cells (PBPC). The occurrence of secondary malignancies, particularly secondary myelodysplastic syndrome/acute leukemias (sMDS/AL), is a critical issue in the autograft setting, and represents a major cause of failure in patients potentially cured after high-dose chemotherapy. However, the reported cumulative incidences of sMDS/AL are quite various, ranging between 3 up to 24%. *Aim of the Study.* To evaluate frequency, actuarial projection, and risk factors of sMDS/AL in a large series of lymphoma patients, homogeneously treated with the high-dose sequential (HDS) chemotherapy approach, with PBPC autograft. *Patients and methods.* The study has been performed on 1088 lymphoma patients treated between 1985 and 2004, at 8 Centers associated to GITIL. All patients received either the original or somewhat modified HDS regimen with autograft; PBPC were usually collected after hd.cyclophosphamide; PBPC harvested after a second mobilization procedure with hd-Ara-C were employed for autograft in a subgroup of patients. The series included 174 patients with HL, and 914 with NHL (561 high-grade and 353 low-grade NHL). Median age was 46 yrs., 633 patients were male. Overall, 551 (51%) patients received HDS as first-line therapy, and 537 patients as salvage treatment following one or more lines of conventional chemo-radiotherapy. Most patients were autografted with PBPC and only few patients received either BM cells alone or BM cells + PBPC. All patients have been monitored with clinical, laboratory and radiological reassessments at given intervals during follow-up. *Results*. At a median follow-up of 5 yrs., the Overall Survival (OS) projections at 5 and 10 yrs. are respectively: 64% and 57% for the whole series, 71% and 65% for patients entering the HDS program at diagnosis, 55% and 46% for those receiving HDS as salvage treatment. Overall, 30 (2.7%) patients developed s-MDS/AL, with a cumulative incidence of sMDS/AL of 3.7% at 5 yrs. Median time of s-MDS/AL occurrence was 2.3 yrs since autograft. In univariate analysis, a higher incidence of sMDS/AL was observed in patients aged >45 y.o. (vs <45 y.o,) and in those receiving PBPC collected at the 2nd mobilization course (vs the 1st collection); none of the other clinical characteristics, including sex, histology, disease status, BM involvement, appeared to be of relevance. *Conclusions*. Overall, the incidence of sMDS/AL observed following HDS is one of the lowest reported so far in lymphoma patients treated with high-dose therapy and autograft. Thus, the use of single agents at high doses does not imply an increased risk of sMDS/AL. In addition, the study indicates that age and type of graft employed for the autografting procedure may be critical for the development of sMDS/AL.

CO-003

GENERATION AND EXPANSION OF DONOR-DERIVED ANTI-LEUKEMIA CTLS IN COMPLIANCE WITH GMP CRITERIA FOR ADOPTIVE IMMUNOTHERAPY AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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In order to pursue the objective of expanding anti-leukemia CTL from leukemia-naïve individuals, we have designed an experimental approach for the *in vitro* generation of anti-leukemia cytotoxic T-lymphocyte (CTL) lines directed against different types of leukemia cells, including AML cells, ALL cells and MDS cells, using CD8-enriched lymphocytes stimulated with dendritic cells (DC), pulsed with apoptotic patient leukemia blasts (LB) as the source of tumor antigens. Effector lymphocytes and DC were derived from allogeneic hematopoietic stem cell transplantation (HSCT) donors. Based on this protocol, IL-7 and IL-12 are added during primary cultures, while IL-2 is added at each leukemia-specific round of

stimulation. We have demonstrated that CTL lines can be generated from CD8-enriched lymphocytes from either HLA-matched or HLA-partially matched HSCT donors, irrespective of the type of malignant cells used for stimulation. Moreover, in order to obtain a large number of anti-leukemia CTLs, to be used for the control of minimal residual disease in transplanted patients, anti-leukemia CTL lines derived after at least two rounds of leukemia-specific stimulation were further expanded using an antigen-independent protocol, using OKT3, IL-2 and irradiated allogeneic feeder cells. We demonstrated that anti-leukemia CTL lines were expanded in the range of 100-200 fold, relative to the number of cells seeded at the beginning of the cultures, without loosing their specificity. In the present study, we have evaluated the feasibility of refining and up-scaling experimental conditions, to meet GMP criteria suitable for use in the clinical setting. In particular we tested the possibility of inducing and expanding a large number of anti-leukemia T cells while preserving their functional and phenotypic features. To translate to the clinical setting, we used serum free Cellgro medium, all reagents were for *in vivo* use, while IL-4, IL-7 and IL-12 belong to dedicated lots. Our results demonstrated that, while up-scaling production and the use of serum-free medium slightly reduced the expansion rate of anti-tumor CTLs (by about 30%) phenotypic and functional characteristics of CTLs were comparable with those obtained under experimental conditions. In particular, we demonstrated that, starting from a mean of fifty million CD8-enriched lymphocytes and ten million leukemia blasts, for each lot of CTLs we can cryopreserve about a billion or more cells, while in total we can reach more than ten billion CTLs. In conclusion, we demonstrated the feasibility of obtaining large quantities of anti-leukemia specific CTLs suitable for *in vivo* use in adoptive immunotherapy approaches. A phase I protocol has been designed and submitted for approval to the competent regulatory agency.

CO-004

DEVELOPMENT OF A QUANTITATIVE REAL TIME PCR ASSAY FOR CHIMERISM MONITORING AFTER SEX-MISMATCHED STEM CELL TRANSPLANTATION

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Relapse is the most frequent cause of failure after allogeneic blood stem cell transplantation (SCT) in patients with nonmalignant and malignant hematological diseases, the detection of hematopoietic chimerism is an useful tool in the follow up of SCT patients to monitor engraftment, for early diagnosis of graft failure or of disease relapse. DNA-based PCR amplification of polymorphic genes, such as nucleotide tandem repeats (VNTR or STR), is the most frequently used method, it has the advantage of being indipendent from sex mismatch but it's expensive and coumbersome because several markers have to be tested in separate reactions, then their ability to discriminate between donor and recipient is limited, and the sensitivity is not higher than 1%. In addition, quantification of the degree of mixed chimerism is sometimes unreliable, since the assay requires diluition. As a consequence, a considerable number of patients relapse notwithstanding complete donor chimerism (CC), likely because the percentage of host cells does not reach the minimum level for detection. Moving from these considerations, we have developed a new method for chimerism detection, based on quantitative real-time PCR (qRT-PCR) determination the SRY gene that has improved sensitivity on STR or VNTR. With this technology we undertook a retrospective analysis in a series of 11 consecutive patients receiving a X -> Y allotransplant at our institution and compared the results with those obtained by STR analysis. PCR was performed using sequence-specific primers and hybridization probes for SRY gene, median number of samples patient was four (range: 3-16). Standard curves for the Y chromosome-specific sequence were generated by a serial diluitions of male DNA in a female background. The sensitivity of assay was 10^{-5} ng, the intraassay and interassay variations were <1% and <1% respectively. There was an excellent concordance with STR-PCR data in the range of 10-1% residual host cells, we observed a correlation coefficient of 0.807 (Figure 1), however STR analysis over-estimated residual host levels lower than 1% while the qRT-PCR was able to detect levels of chimerism up 10^{5} (0.001%) (Figure 2). In conclusion the sensitivity for low chimerism levels and the possibility of quantifying small changes are major advantages of qRT-PCR of SRY gene on STR techniques and holds the promise to be useful for chimerism analysis of SCT. In addition, this procedure has higher accuracy, faster performance, and is less expensivet than STR, the only limitation being represented by the applicability to sex-mismatch.

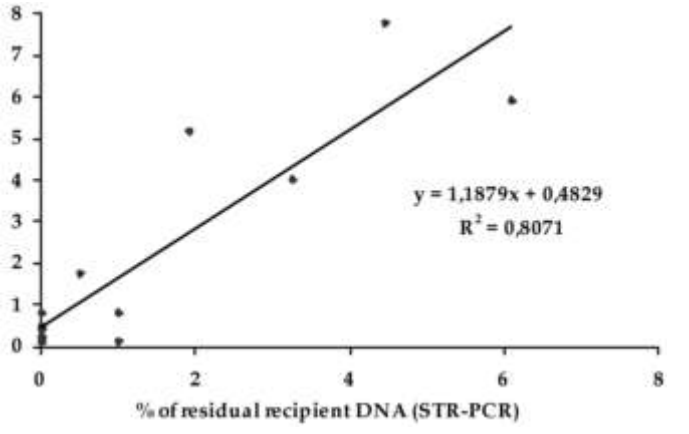

Figure 1.

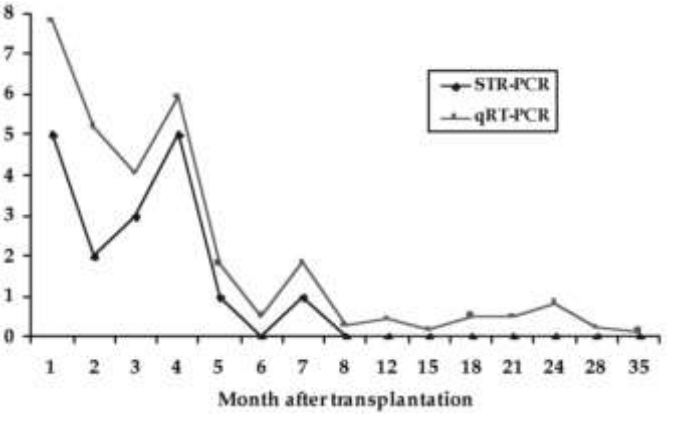

Figure 2.

CO-005

POWERFUL DOWNREGULATION OF LYMPHOCYTE PROLIFERATION BY REGULATORY CELLS GENERATED UPON INTERACTION WITH MESENCHYMAL STEM CELLS

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Bone marrow derived mesenchymal stem cells (MSC) can exert an immunosuppressive effect on lymphocyte proliferation that would be useful in controlling graft-versus host disease. Beside the immunosuppressive effect of MSC due to direct interaction of MSC with lymphocytes, immunosuppression could be determined by an indirect mechanism that is the generation of regulatory cells. This hypothesis havs been tested by co-culturing MSC with allogeneic peripheral blood mononuclear cells (PBMC). We found that at very low MSC:PBMC ratios (1:100-1:500), the interaction between allogeneic PBMC and MSC induced expression on lymphocytes of CD25 and CD69 activation antigens and consequent PBMC proliferation. Triggering of PBMC was accompanied by a strong production of pro-inflammatory and immunoregulatory cytokines, including TNFα, IFNγ, IL1β, IL6, IL10 and detectable levels of pro-proliferating cytokines as IL2 and IL12. Noteworthy, the amount of IL1β, IL10 and IL6 (5000-12000 pg/mLl) was 10-100 fold higher than that detected in supernatant from PBMC triggered with anti-CD3 monoclonal antibodies (mAb) or allogeneic cells (50-1500

pg/mL). On the other hand, similar amounts of IFNγ were found in PBMC-MSC co-cultures and PBMC stimulated with anti-CD3 mAb (range 300-2500 pg/mL, n=4). Binding between PBMC and MSC was needed to induce cytokine production or proliferation as a strong impairment (90-100% of inhibition) was found when PBMC and MSC were separated by a Millicell porous membrane. Indeed, these effects were dependent on the engagement of LFA3 expressed on MSC with CD2 on lymphocytes as a combination of anti-LFA3 and anti-CD2 mAb strongly inhibited PBMC proliferation (75-85%) and cytokine production (65-80%). Importantly, highly purified T cells or CD4+ or CD8+ T cell subsets did not proliferate in response to MSC suggesting that the presence of monocytes, and thus monocyte-derived cytokines as IL1β, were needed to trigger cell proliferation. Indeed, in supernatants derived from co-cultures of monocytes and MSC were found high amounts of IL1-β similar to those derived from monocytes stimulated with LPS indicating that interaction between MSC and monocytes trigger IL1β release. Cell populations derived from MSC-PBMC co-cultures were composed of cells with a short life (7-15 days) and they could not be expanded *in vitro* in the presence of IL2 or IL15. All cells expressed the leukocyte common antigen CD45, the majority was CD2+ (70-90%) with variable proportions (40-75%, n=10) of putative regulatory CD4+CD25+ T cells while a few cells (2-5%, n=6) expressed GITR and intracytoplasmic CTLA4. Cell populations derived from PBMC-MSC co-cultures displayed a functional behaviour of regulatory cells (Regc) as they inhibited mixed lymphocyte reaction (MLR) and CD3-driven lymphocyte proliferation as well as recall antigen stimulation in secondary MLR. This effect was evident at very low numbers of Regc added to cell cultures (70-90% of inhibition at 1:200 Regc:responder cell ratio, n=6). This finding indicate that Regc derived from PBMC-MSC co-cultures exert a stronger regulatory effect than classical CD4+CD25+ regulatory T cells (Treg) expressing CTLA4 and GITR (effectiveness at 1:1-1:10 Treg:responder ratio according to what reported in the literature). Regc with a superimposable inhibiting effect were generated in PBMC:MSC co-cultures also starting from PBMC purged of CD25+ cells but not from highly purified CD4+ or CD8+ lymphocytes. Noteworthy, Regc exerted inhibition of lymphocyte proliferation through the induction of apoptosis of responder lymphocytes (autologous or allogeneic): indeed, in the presence of Regc, the large majority (85-95%) of cells harvested from either MLR or upon stimulation with anti-CD3 mAb expressed activated caspase 8 and 3, DNA fragmentation and morphology of apoptotic cells. These findings indicate that MSC can by themselves trigger activation and proliferation of allogeneic lymphocytes and they induce the generation of functional Regc which in turn are able to down-regulate lymphocyte activation through the induction of programmed cell death.

CO-006

THE EXTRACELLULAR NUCLEOTIDE UTP IS A POTENT INDUCER OF THE MIGRATION OF HEMATOPOIETIC STEM CELLS

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Homing and engraftment of hematopoietic stem cells (HSCs) to the bone marrow (BM) involve a complex interplay between chemokines, cytokines, and non-peptide molecules. Extracellular nucleotides and their cognate P2 receptors are emerging as key-factors of inflammation and related chemotactic responses. In this study, we investigated the activity of extracellular adenosine-triphosphate (ATP) and uridine-triphosphate (UTP) on CXCL12-stimulated CD34+ HSCs chemotaxis. *In vitro*, UTP significantly improved HSCs migration, inhibited CXCR4 down-regulation of migrating CD34+ cells and increased cell adhesion to fibronectin. *In vivo*, preincubation with UTP significantly enhanced the BM homing efficiency of human CD34+ cells in immunodeficient mice. Pertussis toxin blocked CXCL12- and UTP-dependent chemotactic responses, suggesting that G-protein α-subunits (Gαi) may provide a converging signal for CXCR4- and P2Y-activated transduction pathways. In addition, gene expression profiling of UTP-treated CD34+ cells and *in vitro* inhibition assays demonstrated that Rho guanosine 5'-triphosphatases (GTPase) Rac2 and downstream effectors Rho GTPase–activated kinases 1 and 2 (ROCK1/2) are involved in UTP-promoted/CXCL12-dependent HSCs migration. Our data suggest that UTP may physiologically modulate the migration of HSCs and their homing to the BM, in concert with CXCL12, via the activation of converging signaling pathways between CXCR4 and P2Y receptors, involving Gαi proteins and RhoGTPases.

CO-007

THE METASTASIS-ASSOCIATED 67 KDA LAMININ RECEPTOR IS INVOLVED IN G-CSF-INDUCED HEMATOPOIETIC STEM CELL MOBILIZATION

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The 67 kDa laminin receptor (67LR) is a non-integrin cell-surface receptor with high affinity for laminin which plays a key role in tumor invasion and metastasis. We investigated the role of 67LR in granulocyte-colony stimulating factor (G-CSF)-induced mobilization of $CD34^+$ hematopoietic stem cells (HSCs) from 35 healthy donors. G-CSF administration increased 67LR expression in circulating $CD34^+$ cells; by contrast, unstimulated BM $CD34^+$ cells showed very low levels of 67LR. Using as cut off a percentage of circulating 67LR positive CD34+ cells higher than 20%, 67LR expression resulted increased in 31/35 donors: mean percentage ± SEM of circulating 67LR positive $CD34^+$ cells 1.86±0.2% (range 0.5-7%) before G-CSF administration and 46.3±4.1% (range 23-86%) on the day of cell harvesting (day 4 or 5 of G-CSF administration) ($p<0.0001$). The mean percentage ± SEM of 67LR positive unstimulated BM CD34+ cells from 15 normal subjects was 5.1±1.1% (range 1-13%). Therefore, 67LR expression was low during steady-state conditions, when normal $CD34^+$ cells reside in the BM compartment whereas it was up-regulated by G-CSF administration and by transfer into the circulation. In fact, G-CSF withdrawal was associated with a rapid reduction of 67LR expression on $CD34^+$ cells in all G-CSF-treated donors. Noteworthy, 4 out of 5 donors not showing 67LR increase on circulating $CD34^+$ cells after G-CSF treatment mobilized poorly; indeed, they obtained a peak of less than 20 $CD34^+$ cells/10^6;L and did not achieve the target $CD34^+$ cell yield >2×10^6 $CD34^+$ cells/kg in one apheresis procedure after 5 days of G-CSF administration. Accordingly, linear regression analysis showed that both numbers and percentages of 67LR positive circulating $CD34^+$ cells after G-CSF administration directly correlated with $CD34^+$ cell peak values on the day of collection (r=0.7, p=0.001 and r=0.5, p=0.002, respectively). During G-CSF-induced HSC mobilization, the expression of laminin receptors switched from α 6 integrins, which mediated laminin-dependent adhesion of steady-state human marrow HSCs, to 67LR, responsible for G-CSF-mobilized HSC adhesion and migration toward laminin. *In vitro* G-CSF treatment, alone or combined with exposure to marrow-derived endothelial cells, induced 67LR up-regulation in marrow HSCs; moreover, anti-67LR antibodies significantly inhibited transendothelial migration of G-CSF-stimulated marrow HSCs. Finally, G-CSF-induced mobilization in mice was associated with 67LR up-regulation both in circulating and marrow CD34+ cells, and anti-67LR antibodies significantly reduced HSC mobilization, providing the first *in vivo* evidence for 67LR involvement in stem cell egress from bone marrow after G-CSF administration. In conclusion, 67LR upregulation in G-CSF-mobilized HSCs correlates with their successful mobilization and reflects its increase in marrow HSCs, which contributes to the egress from bone marrow by mediating laminin-dependent cell adhesion and transendothelial migration.

Hemostasis and Thrombosis

CO-008

INCIDENCE AND LABORATORY FEATURES OF THROMBOCYTOPENIA IN 43 PATIENTS WITH VON WILLEBRAND DISEASE TYPE 2B: CORRELATION WITH MOLECULAR DEFECTS AND ACQUIRED MODIFICATIONS OF VON WILLEBRAND FACTOR

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Background. Von Willebrand type 2B (VWD) is an inherited bleeding disorder caused by abnormal von Willebrand factor (VWF) that displays enhanced binding to the platelet glicoprotein 1b α (GpIba). VWD 2B is due to a group of mutations clustered within VWF A1 domain and is characterized by heightened binding of its high molecular weight multimers (HMW) to platelets often resulting in moderate-mild thrombocytopenia. Even though there are many reports on thrombocytopenia associated with VWD 2B, retrospective and prospective studies on large cohort of patients are not available. *Aims and design of the study.* to determine incidence and laboratory features of thrombocytopenia in VWD 2B, we have prospectively observed for one year our cohort of 43 patients (18 families) previously characterized by VWF mutations. *Methods.* Data of platelet count with mean platelet volume (MPV) and morphologic evaluation of the blood smear to search for aggregates were associated with physiologic or pathologic conditions such as pregnancy, infections, surgery or use of DDAVP. All patients were characterized by ristocetin induced platelet agglutination (RIPA) in the Platelet Rich Plasma (PRP), ristocetin cofactor activity (VWF:RCo) with VWF antigen (VWF:Ag), multimeric structure of VWF. Mutations within VWF A1 domain were searched for and confirmed by sequencing exon 28. *Results.* Among 43 VWD cases, thrombocytopenia was found at baseline in only 9 (21%), but was observed after stress conditions in additional 38 cases (88%); no reduced platelet counts was found in 9 patients (21%) from two different families. An increased MPV was found in 35 cases but giant platelet and aggregates in only 5 cases. All these phenotypic data were correlated to VWF molecular defects as shown in the Table 1.

Table 1.

				Low Plt (<140x10⁹)		*Plt Morphology*	
Mutation	*RIPA (mg/mL)*	*VWF:Ag (U/dL)*	*Basal (n)*	*Post stress (n)*	*MPV (micron³)*	*gp/aggr.*	*(n)*
R1306W	(15)	0.65	27	4	15	10.3	3
R1308C	(5)	0.72	40	2	5	11.5	2
R1308L	(5)	0.50	37	0	0	9.1	0
I1309V	(6)	0.40	79	2	6	11.8	0
V1316M	(3)	0.50	45	2	3	9.2	1
P1337L	(4)	0.50	39	0	4	9.5	0
R1341Q	(4)	0.67	43	0	0	9.9	0
R1341W	(1)	0.70	43	1	1	9.9	0

Conclusions. Based on these results, thrombocytopenia is associated in most VWD 2B patients especially when high levels of mutant VWF are triggered by physiologic and pathologic stress conditions. However, not all VWD 2B show thrombocytopenia and a relatively high degree of heterogeneity of this phenomenon occurs within patients characterized by the same molecular defects.

CO-009

PERI-OPERATIVE BRIDGING THERAPY WITH LOW MOLECULAR WEIGH HEPARIN IN PATIENTS REQUIRING INTERRUPTION OF LONG-TERM ORAL ANTICOAGULANT THERAPY

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Introduction. The interruption of long-term oral anticoagulant (OAC) therapy for surgery or other invasive procedures puts patients at increased risk of thromboembolic events. Peri-operative 'bridging' anticoagulant therapy may reduce this risk; however, its efficacy and safety has not been established. We conducted a study testing the hypothesis that low-molecular weight heparin is a safe and efficacious perioperative bridging therapy in patients on long-term OACs. *Methods.* This prospective, cohort study included patients requiring bridging therapy due to major surgery (defined as surgery lasting > 1 hour), invasive procedures or minor surgery at increased risk for bleeding. Patients were considered to be at low or at high-risk for thrombosis. The first group was constituted by patients suffering of Atrial Fibrillation without previous Arterial Thromboembolism (AF-noAT), Venous ThromboEmbolism (VTE) lasted more than 3 months, or patients with Prosthetic Aortic Valves (PAV). High-risk group was constituted by patients with Atrial Fibrillation with previous Arterial Thromboembolism (AF-AT), Prosthetic Mitralic Valves (PMV) or patients with recent VTE. In the entire population, Warfarin was discontinued 5 days prior to the surgical procedure. In patients considered at low-risk for thrombosis, LMWH (at prophylactic dosage) was commenced the night before the procedure. In patients considered at high-risk for thrombosis LMWH (at therapeutic dosage) was started 3 days prior to, and continued until the night before the procedure. Warfarin was restarted the evening of the procedure and LMWH (at prophylactic or therapeutic dosage according to the patients' thrombotic risk) was reinitiated 12-24 hours post-procedure and continued until the INR was therapeutic. The primary efficacy endpoint was the incidence of thromboembolism from warfarin cessation to 28 days post-procedure. The primary safety endpoint was incidence of major haemorrhage from first dose of LMWH until 24 hours after the last dose. *Results.* Over a period of 4 years (2001-2005), a total of 228 patients (planned to major and minor surgery or invasive procedures) were included in the study. Conditions requiring long-term OAT were the following: 26 (11.4%) for VTE, 92 (40.3%) for AF-AT, 43 (18.8%) for AF-noAT, 4 (1.7%) for both, 53 (23.2%) for PAV/PMV and 10 (4.3%) for others (arterial hypertension, dilatative myocardiopathy, valvulopathy, myocardial infarction, coronary artery by-pass graft). All patients received LMWH (intention-to-treat group); among them, 132 (60%) belonged to low-risk group and 96 (40%) to high-risk group. In total, 43 (18.8%) underwent major surgery, 58 (25.4%) minor surgery and 127 (55.7%) invasive procedures. Thromboembolic events occurred in 4 patients (3 [3.1%] belonging to high-risk and 1 [0.75%] to low-risk group); 3 events (1 peripheral arterial thromboembolism and 2 transient ischemic attacks) occurred in AF-AT patients, 1 event (pulmonary embolism) occurred in VTE patient (Table 1). Major haemorrhages occurred in 5 patients belonging to high-risk (5.2%) and 1 (0.75%) to low-risk group (Table 1). All major haemorrhage occurred in patients undergoing major surgery; none of the haemorrhages were intracranial, retroperitoneal, or intraocular, were fatal, or required intervention (Table 2). *Conclusion.* Use of LMWH in accordance with the bridging regimen described is feasible and safe in patients undergoing minor surgery or an invasive procedure. Most major haemorrhages occurred in patients undergoing major surgery. Further studies are needed to optimize bridging therapy with LMWH in patients undergoing major surgery.

Table 1. Complications occured in Low- and High-Risk Groups.

	[1]LMWH	*[2]LMWH*
Patients characteristics n (%)	Low-risk group 132 (60%)	High-risk group 96 (40%)
Complications		
TE total n (%)	1 (0.75)	3 (3.31)
Arterial	0	3 (3.1)
Venous	1 (0.75)	0
Bleeding total n (%)	5 (3.8)	12 (12.5)
[3]Major	1 (0.75)	5 (5.2)
Minor	4 (3)	7 (7.3)

[1]Low Molecular Weight Heparin at prophylactic doses (accordingly to manufacturer).
[2]Low Molecular Weight Heparin at therapeutic doses (accordingly to manufacturer).
[3]All occurred during major surgery.

Table 2. Type of surgery related to major haemorrage.

Events	*Major surgery (43)*	*Minor surgery (58)*	*Invasive procedure (127)*
Major hemorrhages (%)	6 (13.9)	0	0
Type of procedure associated with major hemorrhage (n)	TKR (2), THR (2); Abdominal hernia repair (1); Aortofemoral construction (1)	-	-

CO-010

EXPRESSION STUDIES OF TWO MISSENSE MUTATIONS (D141Y AND C275S) IN THE VON WILLEBRAND FACTOR (VWF) PROPEPTIDE ASSOCIATED WITH TYPE 3 VON WILLEBRAND DISEASE (VWD)

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Type 3 VWD is a rare autosomal recessive disorder, characterized by unmeasurable levels of VWF in plasma and platelets (VWF:Ag <0.01 IU/mL). Eight missense mutations have been identified in our laboratory in type 3 VWD patients. Five of them are in the VWF propeptide, essential to promote the VWF multimerization and storage. We performed expression studies of two mutations (D141Y and C275S) to confirm their relationship with VWD. Both patients were compound heterozygous, one carries mutation D141Y and a deletion of 4 nucleotides (2266-2269), the other a C275S and a nonsense mutation (W202X). The missense mutations were introduced into the pSV-WVF vector by site direct mutagenesis. Mutated vectors were expressed in COS-7 cells alone and co-expressed along with the wild type (WT). An ELISA method was used to evaluate the expressed rVWFs. Assuming rVWFWT as 100%, a markedly reduced secretion was detected in cell media, 13.9% for rVWFD141Y and 14.2% for rVWFC275S, while in cell lysates the amount of mutant rVWFs were slightly increased (118.2% and 127%). As expected, hybrid rVWFs (mutants and wild type), mimicking the heterozygous form, resulted in a milder reduced secretion (44.8% rVWFWT/ rVWFD141Y and 39.4% rVWFWT/rVWFC275S), although the amount of hybrid rVWFs were higher in cell lysates (139.5% and 144.3%). Multimer analysis of rVWFs showed that both variants strongly impaired the multimerization process since only dimers were present in the cell media. However, both hybrid rVWFs presented a full set of multimers, similarly to the rVWFWT, suggesting that the mutated rVWF did not compromise the multimerization process of the rVWFWT subunits. In conclusion, these experiments showed that the mutations D141Y and C275S of VWF gene result in a quantitative deficiency of VWF in plasma, due probably to a secretion pathway defect associated with intracellular degradation.

CO-011

INCIDENCE, CLINICAL-LABORATORY FEATURES AND MANAGEMENT OF ACQUIRED VON WILLEBRAND SYNDROME AND OTHER ACQUIRED DEFECTS OF HEMOSTASIS IN A COHORT OF 240 PATIENTS WITH CHRONIC LYMPHO-MYELOPROLIFERATIVE DISORDERS

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Background. Acquired von Willebrand Syndrome (AVWS) is a rare bleeding disorder with laboratory findings similar to those for congenital von Willebrand disease. The actual prevalence of AVWS in the general population is unknown because large prospective studies on this syndrome are not available. Retrospective data showed that AVWS is especially frequent in lympho- (LPD) or myeloproliferative (MPD) disorders. Aims and design of the study: to determine incidence, clinical-laboratory features and management of AVWS and other acquired hemostatic defects, we have sequentially observed for one year our cohort of patients with chronic LPD/MPD. Exclusion criteria were platelet counts <70,000/uL and any therapies, including non-steroid anti-inflammatory drugs. *Methods.* A bleeding severity score derived from a detailed history of 11 symptoms. Screening tests: bleeding time (BT), prothrombin time (PT), partial thromboplastin time (PTT), thrombin time (TT) and, if prolonged, PT-PTT-TT 50:50 mixing tests. Additional specific tests: FVIII/VWF activities (AVWS/HA); platelet nucleotides (acquired storage pool defects, ASPD); silice clotting time (SCT), Russel viper venom time (RVVT), anticardiolipin antibodies (ACA) for lupus anticoagulant-antiphospholipid antibodies (LAC/APA). *Results.* Among 458, 240 patients satisfied the inclusion criteria, with percentual (%) diagnosis of MGUS (38), ET (38), CLL (7), PV-CML-IMF (7), HD-NHL (5), MDS M (2), MM (2) and amyloidosis (1). Results are reported in the Table. In one year, severe mucosal (n=21) and non-mucosal (n=13) bleeds in LPD (n=12) or MPD (10) were treated with DDAVP (n=18), FFP/concentrates (n=4), IVIg (n=10), rFVIIa (n=2). *Conclusions.* AVWS and the other acquired hemostatic defects shown here are not so rare (9/16%) and can be severe in LPD/MPD. An early correct diagnosis should improve morbidity and mortality of patients with bleeding complications in chronic LPD/MPD.

Table.

Features	*Lymphoproliferative*	*Myeloproliferative*	*Total*
Case number (%)	122 (51)	118 (49)	240 (100)
Bleeding score (> 10)	30/122 (25)	18/118 (15)	48/240 (20)
Abnormal screening tests	57/122 (48)	22/118 (19)	79/240 (33)
Acquired defects:	21/122 (17)	38/118 (32)	59/240 (25)
1) AVWS	10/122 (8)	12/118 (10)	22/240 (9)
2) ASPD	0/ 122 (0)	19/118 (16)	19/240 (8)
3) LAC/APA	8/ 122 (7)	3/118 (3)	11/240 (5)
4) anti FVIII or X inhibitors	3/122 (2)	4/118 (3)	7/240 (3)

CO-012

HOMOZYGOSITY FOR JAK2V617F IDENTIFIES MPD PATIENTS WITH A MORE SYMPTOMATIC DISEASE A RETROSPECTIVE STUDY ON 989 PATIENTS FROM THE GIMEMA-MPD WORKING PARTY

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An acquired mutation in the JAK2 gene is found at different rates in patients with chronic myeloproliferative disorders (MPD); in about 20-30% of polycythemia vera (PV) or idiopathic myelofibrosis (IM), and in less than 3% of essential thrombocythemia (ET), the mutation is harboured in the homozygote status. This low frequency of homozigosity has prevented until now the elucidation of its impact on disease phenotype. The aim of this study was to evaluate whether homozygosity for JAK2V617F would allow to identify a subgroup of MPD patients with unique clinical characteristics. In an Italian cooperative GIMEMA retrospective study, 989 MPD patients were enrolled from 11 hematology centers. The diagnosis of PV was made in 328 (33%), of ET in 400 (40%), while 224 (23%) were IM and 37 (4%) were post-PV/ET forms (PP/PTMM) of myelofibrosis. Diagnosis of PV or ET was made accordingly to either the PVSG or WHO criteria, while the Consensus Conference Criteria were used for IM. The only eligibility criteria for inclusion was the availability of a JAK2V617F mutational status determination according to the ASO-PCR and the BsaXI digestion method (Baxter, Lancet 2005). In this series, 317 patients (32%) were wild-type (WT), 520 (53%) were JAK2V617F heterozygote and 152 (15%) homozygote; among the latter, 81 were PV, 8 ET, 45 IM and 18 PP/PTMM, accounting for 25%, 2%, 20%, and 49%, respectively, of patients within each diagnostic group. Irrespective of their diagnosis, homozygote patients showed higher leukocyte count and hematocrit level, while platelets were not different from other groups. The frequency of splenomegaly progressively increased from 40%, to 51% to 69% in WT, heterozygotes or homozygotes; similarly, the occurrence of pruritus rose from 8% to 18% to 28%, and that of systemic symptoms from 25% to 30% to 38%. There were 351 thrombotic events, of which 251 were major events and 187 of the microvessels; major hemorrhages were 45. There was a higher incidence of thrombosis in homozygotes (55%) than in heterozygotes (36%) or WT (26%) patients, while there was no difference in hemorrhages. In PV and ET, homozygosity was associated with a greater risk of evolution into myelofibrosis (12% and 25%, respectively) compared to heterozygosity (2.2% and 2.5%); noteworthy, the highest frequency of homozygosity was recorded among PP/PTMM patients (49%). Finally, the frequency of patients overexpressing PRV-1 gene was greater among homozygotes (89%) than heterozygotes (69%) or WT (42%). In conclusion, this large survey of MPD patients, that allowed to evaluate 152 homozygote patients, supports the contention that the loss of wild-type JAK2 allele in hematopoietic cells characterizes a quite homogeneous category of patients with more symptomatic disease within each MPD diagnostic category. Assessment of JAK2V617F homozigosity may have a role in risk prediction and patient management.

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CO-013

THE LEVELS OF JAK2 V617F RNA DICTATE THE CLINICAL PHENOTYPE IN POLYCYTHEMIA VERA AND IDENTIFIES PATIENTS WITH MORE SYMPTOMATIC DISEASE.

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Background. The occurrence of an unique JAK2^{V617F} mutation in phenotypically distinct chronic myeloproliferative disorders (MPD), including polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IM), suggests that other genetic events/gene modifiers might be involved. AIMS. As an approach to unravel significant associations between phenotype and the JAK2 mutation, we have correlated the levels of JAK2^{V617F} RNA with clinical and laboratory characteristics at the diagnosis in 63 patients with polycythemia vera (PV) and 115 with essential thrombocythemia (ET), as diagnosed according to the WHO criteria. METHODS. Wild-type and mutated JAK2 RNA levels were determined by an amplification-refractory mutation sequencing (ARMS) PCR assay on granulocytes, and expressed as the percentage of mutated JAK2 RNA over total (Vannucchi AM *et al,* Leukemia 2006 Mar 30). RESULTS. 53/63 PV patients (84%) and 76/115 (66%) presented detectable levels of JAK2^{V617F} RNA; the amount of mutated RNA was higher in PV than in ET granulocytes (median 52% and 12.5%, respectively; $p<0.0001$). In PV patients, the hematocrit and white blood cell count were significantly related to the amount of mutated RNA, while there was an inverse relationships with MCV and platelet count. None of these parameters significantly correlated with mutated RNA levels in ET. Even when the analyses were restricted to those PV patients who showed RNA levels in a range similar to that observed in ET (1-55%) the above correlations were mantained, thus ruling out that these effects might be simply ascribable to the overall higher load of JAK2^{V617F} RNA in PV than in ET patients. Among PV patients with JAK2^{V617F} mutation, the frequency of splenomegaly, of therapy (flebotomies and chemotherapy) and of chemotherapy requirement were all significantly increased over wild-type patients, but again not in ET pts. On the other hand, in both PV and ET JAK2^{V617F} mutated patients there was a greater frequency of EEC and overexpressed PRV1 gene, while there was no difference in CD34^{+} cell count in the peripheral blood. The percentage of *high-risk* patients was higher among mutated than wild-type ones (63% vs 27%, $p=0.003$) if patients were all considered together, but did not reach the significance level in the ET group alone (62% vs 38%, $p=0.07$); on the contrary, in PV there was a progressive increase in the percentage of high-risk patients according to the amount of mutated RNA (10% in wild-type, 24% in patients with 1-25% JAK2^{V617F} RNA and 66% among those showing 26-100% JAK2^{V617F} RNA). *Conclusions.* By quantifying the amount of JAK2^{V617F} RNA in granulocytes, we documented a gene dosage-effect in PV, but not in ET, suggesting that the JAK2 mutation dictates the clinical phenotype in PV patients while additional genetic or host factors modulate the disease presentation in ET. Also of note, the levels of JAK2^{V617F} RNA identified PV patients with more symptomatic disease in terms of blood abnormalities, therapy requirement and *high-risk* category.

CO-014

CHROMOSOMAL ABNORMALITIES IN MULTIPLE MYELOMA PLASMA CELLS AND THEIR CORRELATION WITH IMMUNOPHENOTYPE

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In the present study we have investigated the relationship between the immunophenotypic profile of myelomatous plasma cells and their specific genetic features by interphase flourescence in situ hybridization (FISH). Between August 2002 and April 2006, 554 consecutive patients with MM, referred to our Hematology Department, entered our study: 350 were at diagnosis and 204 at relapse. FISH analyses were performed on bone marrow plasma cells (BMPC) purified using anti-CD138-coated magnetic beads (Miltenyi Biotech GmbH, Germany) and the purity, assessed by flow cytometry with anti-CD38PE monoclonal antibody (MoAb), always exceeded 90%. Nuclei from fixed PC were prepared for interphase FISH using standard methods. This study was done using DNA probes (Vysis, Downers Grove, IL) to detect chromosome 13 abnormalities, t(4;14)(p16;q32), t(11;14)(q13;q32) and deletion of p53 on chromosome 17. Normal cut-off values for each probe were determined in 200 BMPC intephase nuclei from 10 healthy individuals. We defined a cut-off value of 8% abnormal cells for deletion of chromosomes 13 and 17, and 15% for 14q32 translocations involving the IgH locus, considering both single and dual fusion signals obtained with *dual color dual fusion* probes. These values represent the mean plus 3SD of abnormal cells detected in normal controls. In 96% of all patients at least one numerical alteration of the mentioned chromosomes was found. Del13 was identified in 50.3% of patients. No difference in the prevalence of del13 was observed according to age, serum β-2 microglobulin, clinical stage and immunoglobulin isotype. The frequence of del13 was significantly higher in female (50% vs 35.9%; $p=0.0008$). A significant correlation between del13 and poor prognosis) was found by the analysis of clinical features of 201 patients ($p=0.02$). p53 gene deletion was detected in 15.5% of 84 patients, t(11;14) in 16.5% of 91 patients and t(4;14) in 27% of 174 patients. The identification and quantification of BMPC at diagnosis and their immunophenotypic characteristics were assessed using a flow cytometry approach on whole blood. PC can be easily identified by their strong reactivity to anti-CD38 MoAb and their specific expression of CD138. The immunological phenotype of BMPC was assessed using triple or quadruple combinations of MoAbs for the detection of the following antigens: CD56, CD45, CD40, CD19, CD20, CD52, CD117, cytoplasmic immunoglobulin light chains kappa/lambda. PC carrying del13 showed a statistically significant lower expression of CD45 than PC without del13 (22% vs 36.7%; $p=0.0002$). Moreover, PC with del13 were more frequently CD56 negative (64.5% vs 71%; $p=0.03$). We also observed that patients with PC carrying del13 had a significantly higher BMPC infiltration (16.6% vs 12.2%; $p=0.0003$). PC carrying del p53 were less frequently CD52+ and had a significantly higher BMPC infiltration (25.2% vs 16.3%; $p=0.035$). The presence of t(11;14) has been associated with CD20 expression ($p=0.0002$) and with a lower expression of CD56 ($p=0.04$) on PC surface. Del13 was more frequently found in patients carrying t(4;14) than in patients without this translocation ($p=0.05$). It has been also detected a significat correlation between t(4;14) and the absence of expression of surface monoclonal immunoglobulins ($p=0.036$). A cytogenetic classification of MM subgroups will be useful to more accurately stratify patients into prospective therapeutic trials.

CO-015

CKS1B OVER-EXPRESSION SIGNIFICANTLY PREDICTS FOR A LOWER RATE OF RESPONSE TO PRIMARY THERAPY WITH THALIDOMIDE-DEXAMETHASONE FOR NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS

Terragna C, Renzulli M, Testoni N, Montanari E, Nicci C, Tosi P, Zamagni E, Tacchetti P, Perrone G, Ceccolini M, Cangini D, Toccafondi M, Brioli AM, Martinelli G, Baccarani M, Cavo M

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In Multiple Myeloma (MM) tandem duplication and jumping translocations of the q21 band of chromosome 1 are acquired during progression of the disease and can lead to chromosome (chr.) 1q amplification. In several studies, the amplification of chr. 1 has been linked to poor prognosis after both conventional and high-dose chemotherapy. Recently, a subset of genes mapping on 1q21 band has been identified, whose increased expression may be due to increased DNA copy number. Among these genes, CKS1B has been one of the most significantly upregulated. CKS1B regulates SCF skp2 mediated ubiquitination and proteolysis of the cyclin dependent kinase inhibitor p27(e)Kip1. Low p27(e)Kip1 expression has been reported to be an independent adverse prognostic factor in patients with MM. Aim of the present study was to investigate the relationship between the expression of CKS1B and response to primary therapy with thalidomide and dexamethasone in a large series of patients with newly diagnosed MM. Secondary endpoint was to explore the relationship between the expression of CKS1B and chromosome 13 deletion (del(13)), as assessed by FISH analysis, and t(4;14), as evaluated using a validated RT-PCR assay designed to detect the presence of IgH/MMSET fusion gene. A total of 132 patients were analyzed. The presence of t(4;14) and CKS1B expression were investigated in all patients, while del(13) was studied in 129/132 patients. CKS1B expression was evaluated by Real-time RT-PCR, using GAPDH as internal control. CKS1B values were separated in four different quartiles, with expression levels increasing progressively from quartile 1 to 4. Response to therapy was evaluated according to the criteria proposed by Bladè *et al.*. The Fisher test and the Mann-Whitney test were applied for statistical analysis. On an intent-to-treat basis, the overall probability to respond (≥partial response) to up-front thalidomide-dexamethasone therapy was 71%, while 38 patients (29%) either did not respond or progressed. Median CKS1B expression value was significantly higher in nonresponders in comparison with patients who attained at least a partial response: 1.42 (range 0.15-52.35) vs. 0.89 (range 0-11.88), respectively (p=0.01). In particular, the proportion of patients with no response or progression in the CKS1B expression quartile 4 was significantly higher as opposed to the frequency of nonresponders in the CKS1B expression quartiles 1 to 3 (45.5% vs. 23.2%, respectively; p=0.02). CKS1B over expression did not correlate with the presence of t(4;14) or del(13). Only 6 patients harbouring t(4;14) fell into the CKS1B expression quartile 4, as opposed to 32 patients included into the CKS1B expression quartiles 1-3 (18.2% vs. 32.3%; p, not significant). Similarly, the frequency of del(13) was comparable in the CKS1B expression quartile 4 and in the CKS1B expression quartiles 1-3 (34.4% vs. 44.3%; p, not significant). Likewise, only 2 patients carrying both t(4;14) and del(13) fell into the CKS1B expression quartile 4, as opposed to 15 patients who fell into the CKS1B expression quartiles 1-3 (6.5% vs. 15.3%; p, not significant). In conclusion, in patients with newly diagnosed MM, CKS1B over expression at baseline predicts for a significantly lower probability of response to primary remission induction therapy with thalidomide and dexamethasone. Poor response to thalidomide-dexamethasone conferred by CKS1B over expression is independent from the presence of t(4;14) and/or del(13).

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CO-016

THE CANDIDATE TUMOR SUPPRESSOR ING4 REGULATE IL-8 PRODUCTION IN HUMAN MULTIPLE MYELOMA CELLSAND IT IS INVOLVED IN THEIR PRO-ANGIOGENIC PROPERTIES

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The induction of angiogenesis by multiple myeloma (MM) cells suggests that this process has a critical role in the growth and survival of MM cells. The molecular mechanisms underlying the regulation of angiogenesis in MM have not been completely elucidated. The new candidate tumor suppressor gene inhibitor of growth family member 4 (ING4) has been recently implicated in solid tumors as a repressor of angiogenesis and tumor growth through the association with NF-kB and the suppression of angiogenic related molecules including IL-8 (CXCL8). In this study first we evaluated the mRNA expression of ING4 and of the potential angiogenic related molecules in several human myeloma cell lines (HMCLs): U266, RPMI-8226, XG-1, XG-6, OPM-2, NCI-H929, ARP-1 and JJN3, in EBV+ lines HS-SULTAN and ARH-77 and in purified CD138+ plasmacells (purity >90%) obtained from either 40 MM patients at the diagnosis (stage I-III) or 11 patients with MGUS by both qualitative RT-PCR and quantitative real time PCR. Data obtained were also compared with gene expression profiling ones by microarray analysis (Affimetrix) on a larger database of MM and MGUS patients. Healthy CD20+ BM B lymphocytes and CD138+ plasma cells obtained from reactive tonsils have been used as controls. ING4 has been checked at protein level by western blot and immunoprecipitation on nuclear extracts using a polyclonal anti-p29ING4 Ab. ING4 level has been compared with IL-8 and other pro-angiogenic factors in HMCLs and in MM patients also evaluating its potential correlation with bone marrow angiogenesis. To further evaluate the role of ING4 we used small interfering RNA (siRNA) to transfect and to silent ING4 in MM cells and we evaluated its effect on the production of angiogenic molecules VEGF, Ang-1 and HIF-1α with its target genes NIP3 and AK-3 as well as the pro-angiogenic properties of MM cells in an experimental *in vitro* model (Angiokit). The presence of ING4 transcript was observed in HMCLs even if ING4 mRNA levels were significantly reduced in all HMCLs tested as compared to controls with a median δCt ING4 (δCt ING4-Ct δAbl: 3.2 vs. 0.45 (p<0.05). Consistently a down-regulation of ING4 protein has been observed in all HMCLs tested compared to controls. Similarly to HMCLs, when we checked MM patients we found that CD138+ MM cells had significantly reduced levels of ING4 mRNA as compared to normal plasma cells (median δCt ING4: 3.7 vs. 0.47, p=0.01) with a mean n-fold expression of 0.11 normalized to controls whereas the difference of ING4 expression as compared to MGUS subjects did not reach a statistical significance. Gene expression profiling in a larger cohort of patients confirmed that ING4 expression was not significantly different in MM patients as compared to MGUS. ING4 protein levels were detected in nuclear extracts of CD138+ MM cells at lower level as compared to normal B and plasma cells. The presence of potential ING4 DNA mutation in HMCLs was evaluated by denaturing high-performance liquid chromatography as well as we performed microsatellite analysis on chromosome 12p12-13 that covered a relatively wide chromosomal area including ING4 gene to examine potential allelic loss of ING4. However, any ING4 DNA mutation or loss of heterozygosity was not found in HMCLs and MM cells that could explain the down regulation of ING4 expression. A significantly negative correlation was observed between ING4 and IL-8 mRNA levels in HMCLs (R=-0.84, Spearman 2-tailed test, p=0.04). The trasfection with ING4 siRNA, but not with the non-specific control siRNA, suppressed ING4 mRNA expression in HMCLs leading to a strong up-regulation of IL-8 mRNA (RPMI-8226: ΔCt IL-8: 2.3 vs. 0.1, Δ/ΔCt: 4.92) and IL-8 protein secretion (RPMI-8226: 126±4.3 vs. 365±14.3 pg/mL: p<0.001). Moreover, we found that siRNA anti ING4 affected HIF-1α activity and the expression of its target genes NIP3 and AK-3 by HMCLs in hypoxic condition, whereas had not effect on VEGF, Ang-1 HGF, OPN expression. *In vitro* data has been confirmed by the finding that IL-8 median levels were significantly higher in BM plasma

of MM patients as compared to control subjects (39,3 vs. 25,2 pg/ml p=0.05) and correlated with IL-8 mRNA levels in CD138+ MM cells (R=0.77, p0.041). Interestingly, we observed that MM patients with higher BM IL-8 levels (> 25 pg/mL) have a significantly lower ING4 mRNA levels (median δCt ING4: 4.71 vs. 1.9; p=0.04). Finally, we found that BM plasma of MM patients with higher IL-8 levels significantly stimulate vessel formation as compared to those with low IL-8 levels as well as HMCLs trasfected with siRNA anti ING4 as compared to the control. Consistently MM patients with high microvalscular density (MVD>30) and number of vessels X field have significant lower ING4 mRNA levels as compared to those with low MVD (<30) (median δCt ING4: 4,4 vs. 2.4; p=0.04). In conclusion, our data indicate that the tumor suppressor ING4 is down-regulated in MM cells and regulate IL-8 production modulating the pro-angiogenic properties of MM cells.

CO-017

EFFECTS OF HUMAN MYELOMA CELLS ON WNT SIGNALING IN HUMAN BONE MARROW OSTEOPROGENITOR CELLS

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The mechanisms by which multiple myeloma (MM) cells inhibit osteoblast and bone formation are not completely clear. Recently we have demonstrated that human myeloma cells block the critical osteogenic transcription factor RUNX2 in osteoprogenitor cells inhibiting osteoblastogenesis. However several data indicate that Wnt signaling is also involved in the regulation of osteoblast formation either through its canonical pathway, or at least in part through its non-canonical one. Canonical Wnt signaling pathway is mainly activated by autocrine or paracrine Wnt 1/3a that interact with Lrp5-6/Fzd receptor complexes and induce the stabilization and nuclear translocation of β-catenin. Rising of nuclear active β-catenin in turn affects gene expression through the activation of the transcription system Lef1/TCF. Given that MM cells produce the WNT inhibitor DKK-1 that correlates with the presence of bone lesions, we have investigated the potential effects of human myeloma cells on Wnt signaling in human bone marrow (BM) osteoprogenitor cells and its role in the pathophysiology of MM. First we checked several human myeloma cell lines (HMCLs) (XG-1, XG-6, RPMI-8226, U266, OPM-2, JJN3, NCI-H929, ARP-1) and purified CD138+ MM cells obtained from 40 newly diagnosed MM patients for the expression of the WNT inhibitors DKK-1 and secreted Frizzled-related proteins (sFRP)-1,-2,-3,-4 finding that 50% of HMCLs and 70% of MM patients were positive for DKK-1. A similar pattern of expression was observed for sFRP-3 whereas MM cells were negative either for sFRP-2 with the exception of U266 or for sFRP-1 and sFRP-4. Following, we performed a co-culture system with BM osteoprogenitor cells (PreOB), obtained after two weeks of differentiation and MM cells, in the presence or absence of a transwell system for 12-72 hours. After the co-culture period in the cell-to-cell contact condition MM cells were depleted to avoid their contamination. In parallel conditions the effect on osteoblast differentiation and Wnt signaling of BM plasma of MM patients will be tested. WNT signaling pathway was analyzed in PreOB by microarray using a specific Oligo GEArray kit and further evaluated by RT-PCR and western blot analysis on selected molecules. The presence of Wnt receptors Fzd was demonstrated in human osteoprogenitor cells. Any effect on the expression of DKK-1,-2,-3,-4 as well as sFRP1,-2,-3,-4 as well as of Wnt pathway mediators by PreOB was not observed at mRNa levels by the array. Results were also confirmed for DKK-1 and sFRPs by western blot analysis. The study of β-catenin signaling was deepened in both HMCLs and PreOB using either specific antibodies for the active de-phosphorylated form and inactive phosphorilated one or ELISA assay to evaluate the total levels of β-catenin in both cytosolic and nuclear extracts. Finally, β-catenin expression was evaluated on bone biopsies by immunohistochemistry in MM patients and correlated with the presence of bone lesions. A positive correlation between DKK-1/sFRP-3 expression and the presence of active β-catenin has been found in MM cells. Osteoblast differentiation in BM PreOB was associated with a slight increase of nuclear and cytosolic levels of β-catenin after 2-3 weeks of differentiation. However, we failed to observe an inhibitory effect on nuclear levels of active β-catenin and total β-catenin as well as on the transcription factors LEF-1 and TCF in PreOB after co-culture with HMCLs or the majority of fresh purified MM cells either in presence or absence of BMP-2. In line with these observations, we found that Wnt3 stimulation did not restore the inhibitory effect on bone nodule formation induced by HMCLs as well as DKK-1 and sFRP-3 inhibited bone nodule formation and active β-catenin in BM PreOB only at high concentration (>500 ng/mL). Consistently we found that BM plasma of MM patients did not inhibit Wnt signaling in human PreOB independently on the expression of DKK-1 or sFRP-3 showing a median sFRP-3 and DKK-1 level of 5.92 ng/mL and 20 ng/mL, respectively. Finally we failed to find a correlation between the presence of bone lesions and β-catenin expression by stromal/osteoblastic in MM patients. On the contrary, when we checked the effect of MM cells or BM plasma of MM patients on Wnt signaling in murine stromal cell lines MC3T3 in presence of BMP-2 we found an inhibition of the active β-catenin levels and an increase of the inactive phosphorilated one suggesting that MM cells may affect in Wnt signaling in murine but not in human system. Our data indicate that other mechanisms rather than Wnt inhibition could be involved in DKK-1 mediated bone destruction in MM.

CO-018

GENE EXPRESSION PROFILING OF MYELOMA CELLS TO PREDICT ATTAINMENT OF (NEAR) COMPLETE RESPONSE TO PRIMARY THERAPY WITH THALIDOMIDE-DEXAMETHASONE FOR NEWLY DIAGNOSED MULTIPLE MYELOMA

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In recent years, treatment paradigm of targeting the *soil* bone marrow microenvironment as a means of interfering with the growth of the myeloma (MM) *seed* has provided the rationale for investigational clinical trials of novel agents combined with old drugs in an attempt to enhance cytotoxicity, reverse drug resistance and maximize tumor response. Thalidomide, an old drug with an infamous past, has redeemed itself as an effective treatment strategy for relapsed/refractory MM and actually represents a standard of care also for patients with newly diagnosed disease. Our group has recently provided demonstration that thalidomide in combination with dexamethasone as front-line therapy in preparation for autologous transplantation is superior to VAD in terms of increased rate of response (≥ partial response: 76%) and magnitude of tumor reduction. In particular, the probability to attain at least a very good partial response (VGPR) was 19%, including 13% of patients in complete response (CR) or near complete response (nCR). In the present study we adopted a Gene Expression Profiling (GEP) strategy in an attempt to identify a signature able to predict the probability to attain ≥ nCR to combined thalidomide-dexamethasone as upfront therapy for patients with newly diagnosed MM. Bone marrow samples obtained at diagnosis from patients enrolled in the *Bologna 2002* clinical trial including thalidomide-dexamethasone and double autologous transplantation were used throughout the study. Overall, 32 patients who were evaluable for response to thalidomide-dexamethasone in preparation for autologous transplantation were included in the present analysis. GEP was performed using the Affymetrix HG133 Plus microarray platform as per manufacturer's recommendation. The Affymetrix output (CEL files) was imported into Genespring 7.3 (Agilent technologies) microarray analysis software, where data files were normalized across chips using GCRMA and to the 50th percentile, followed by per gene normalization to median. Criteria of response were those established by Bladè *et al.*, with the addition of a VGPR and nCR categories. At first, we used the first set of chips obtained from 16 patients as training set, in order to identify the gene signature and the ≥ nCR predictor genes. Thereafter, we used a different set of chips obtained from 16 patients non related to the training set as test set, in order to confirm the results and to check the predictive strength of the ≥ nCR predictor genes. Overall, six of the 32 patients (19%) obtained at least a nCR to thalidomide-dexamethasone, whereas the remaining 26 patients either achieved a partial response or did not respond. In particular, both the training and the test sets included 3 patients who attained ≥ nCR. We identified a gene signature of 61 genes, able to significantly distinguish patients with ≥ nCR from the others (p=0.01). Moreover, using the class prediction tool available in Genespring (Support Vector Machines), we identified 25 genes that reliable predicted the probability to attain ≥ nCR to primary thera-

py with thalidomide and dexamethasone. These results could be the first step to create a custom array or to adopt microfluidic cards using a small number of genes, in an attempt to select at diagnosis patients who will respond very favourably to a particular treatment strategy.

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CO-019

MEK1 INHIBITION SENSITIZES MULTIPLE MYELOMA TUMOR CELLS TO ARSENIC TRIOXIDE (ATO)-INDUCED APOPTOSIS

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Although Arsenic Trioxide (ATO) is an effective therapy in acute promyelocytic leukemia (APL), its use in other malignancies is limited by the toxicity of concentrations required to induce apoptosis in non-APL tumor cells. Preliminary data from clinical studies indicate that ATO has clinical activity as a single agent in human multiple myeloma (MM) but therapeutic effects are not close to APL success, in particular in the setting of advanced and chemoresistant disease studied. Taken these values, combination therapies are being investigated. We recently reported that PD184352 (PD) (kindly provided to us by Dr J. S. Sebolt-Leopold, Pfizer Global Research & Development, Ann Arbor, MI), a highly selective inhibitor of MEK1 phosphorylation and activation, strikingly enhances ATO-mediated apoptosis in NB4, K562 cell lines via p73-p53AIP1 pathway activation (Blood 104: 519-525, 2004), and in primary Acute Myelogenous Leukemia (AML) via Bad, p73-p53AIP1 and p53 pro-apoptotic pathways activation (Blood 107: 4549-4554, 2006). The aim of this study was to investigate whether the combination PD plus ATO has cytotoxic effects on MM cells. Apoptosis was evaluated, after 48 hours of treatment, by measurement of sub-G1 DNA content, annexin V binding and mitochondrial transmembrane potential assays. We first analyzed the pharmacologic interactions between PD and ATO using a fixed-ratio experimental design on 8 human myeloma cell lines (HMCL) with varying p53 status (RPMI 8226, U266, OPM2, XG-1, XG-6, JJN3, HS-SULTAN, NCI-H929) and found that the combined treatment resulted in the synergistic (Combination Index <1.0) induction of apoptosis in NCI-H929, XG-1, XG-6, RPMI 8226, OPM2, SULTAN and JJN3 HMCL. Conversely, the combination of PD plus ATO had a slightly antagonistic effect in U266 HMCL (Combination Index >1). The dual treatment PD plus ATO induced caspase-3 and polyadenosine diphosphate-ribose polymerase (PARP) degradation in all the responsive HMCL. Moreover, neither co-culture with bone marrow stromal cells nor IL-6 stimulation (20 ng/mL) protected against PD plus ATO-induced cytotoxicity. Subsequently, in order to test whether freshly isolated myeloma cells behave as HMCL, CD138+ MM cells from the bone marrow of MM patients were cultured for two days with pharmacological concentrations of ATO (1-2 μM), PD (2 microM), or both and apoptosis was scored after 24 and 48 hours of treatment. The treatment with PD significantly (p<.01) enhanced the apoptosis induced by ATO in 70% of MM analyzed cases (n=10). Importantly, PD treatment significantly attenuated (p<.01 n=2), or did not affect (n=1), the ATO cytotoxicity in normal B cells. In conclusion, these findings suggest that a strategy combining ATO with disruption of MEK pathway warrants attention in MM and could become an effective therapeutic strategy for the treatment of MM.

CO-020

BIM PROMOTER IS EPIGENETICALLY SILENCED IN MALIGNANT LYMPHOID CELLS, LEADING TO DOWNREGULATION OF BIM EXPRESSION AND PROTECTION FROM

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Bim is a proapoptotic BH3-only, Bcl-2 family member. It exerts its proapoptotic activity by two different mechanisms: directly, by activating the proapoptotic protein Bax, leading to the permeabilization of the mitochondrial outer membrane and indirectly, by inhibiting the anti-apoptotic Bcl-2 family members, mostly Bcl-XL and Mcl-1. Gene knock-out studies indicate that Bim is the major physiological antagonist of the prosurvival Bcl-2 proteins in B and T lymphocytes; it is also essential for the development of T cells, for the induction of apoptosis in activated T cells following an immune response and to prevent autoimmunity. In a Bim knock-out mouse the loss of both alleles led to a marked protection from several proapoptotic stimuli in pre-T cells. Notably, the protective effect in the Bim+/- mouse was intermediate between Bim-/- and Bim+/+, thus showing a *gene dosing* effect. We performed a CpG Islands prediction analysis on Bim promoter, identifying a putative CpG Island. Using a Bisulfite Modification-Clonal Sequencing Analysis (BMCSA), we investigated the methylation status of 19 CpG sites (from nucleotide –504 to +64 from the ATG start site) in the Bim promoter in 12 malignant hematological cell lines: 6 of lymphoid and 6 of myeloid origin. A minimum of 6 clones were analyzed. An homogeneous, very high level of methylation was present in all the lymphoid cell lines (Average Level of Methylation (ALM) 93.4±4.4% Standard Deviation [SD]) and a variable level of methylation in the myeloid counterpart (ALM 37.1 ±32.4%). In agreement with *in vitro* data, we have found *in vivo* evidence of lymphoid Bim promoter methylation in frozen tumor samples from patients affected by NPM/ALK positive anaplastic large cells lymphomas and a very low level of methylation in BCR/ABL positive samples from chronic myeloid leukemia patients. Furthermore, to confirm the specific association of Bim promoter methylation to lymphoid tumors, we analyzed the methylation pattern of normal lymphocytes derived from healthy donors, identifying a very low level of Bim methylation. Treatment of cell lines with the demethylating agent 5-azacytidine (AZA) led to demethylation of Bim promoter and to a potent induction of Bim at mRNA and protein level. In the lymphoid, NPM/ALK positive, SUD-HL-1 cell line, in which a complete demethylation (from 100% to 0%) was achieved, the increase in Bim mRNA was 7.7-fold and this correlated with a potent induction of apoptosis, as assessed by TUNEL and Annexin V assays. Similar results were obtained using a different demethylating agent: 5-aza-2'deoxycytidine. To demonstrate if Bim promoter methylation is an active process, we performed a 5-days wash-out experiment on SUDHL-1 cell line previously treated with AZA or DAC. We showed that the demethylation is reversible and that following remethylation the expression of Bim at mRNA and protein level is reduced back to the initial value, suggesting the presence of an active mechanism. To assess whether Bim CpG island hypermethylation is linked to chromatin condensation (heterochromatin), which is common in highly silenced genomic regions, we performed anti-acetylated-Histone-H3 chromatin immunoprecipitation (ChIP) analyses. It is widely accepted that loss of acetylation on Histone H3 tails leads to chromatin condensation. Deacetylation of Histone H3 is thus commonly used as a specific marker for the presence of heterochromatin. ChIP analyses on SUD-HL-1 (high methylation level) and Lama-84-R (low methylation level) cell lines revealed a dramatic reduction of the acetylation level in SUD-HL-1 compared to Lama-84-R, indicating that methylation of Bim CpG Island is associated with loss of acetylation on Histone H3 tails and thus to chromatin condensation. To assess whether deacetylation of Bim promoter correlates with gene silencing, we treated SUD-HL-1 (deacetylated) and Lama-84-R (acetylated) cell lines with Tricostatin-A (TSA), a potent inhibitor of Histone Deacetylases (HDAC). This led to a 16.7-fold induction of Bim at mRNA level in SUD-HL-1, as assessed by Real-Time PCR, and correlated with induction of apoptosis, as assessed by TUNEL and Annexin V assays. As expected, no increase in Bim expression could be detected in Lama-84-R cell line. In conclusion, we identified a new regulatory mechanism for the proapoptotic Bim gene, based on epigenetic modifications: histone deacetylation and CpG island hypermethylation actively silence Bim expression in leukemia/lymphomas of B and T origin and this silencing is associated with protection from apoptosis.

Molecular Oncohematology I

CO-021

PRION-LIKE DOPPEL GENE (PRND): A NEW MOLECULAR MARKER POTENTIALLY INVOLVED IN LEUKEMOGENESIS

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The PRND gene encodes Doppel (Dpl), a protein that is strongly expressed in testis and at much lower levels in other tissues. Despite the recent discovery of Dpl involvement in spermiogenesis and in apoptotic death of cerebellar neurons, the physiological role of this prion-like protein remains unknown. Recently, we observed a weak Dpl expression in normal CD34+ bone marrow cells, whereas high levels of PRND transcripts were detected in leukemic cell lines as well as in bone marrow cells from patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). In order to clarify the clinical and biological relevance of Dpl overexpression in these disorders, we searched for possible correlations among Dpl expression, some biological parameters and clinical-pathological features. Moreover, in some MDS patients we sequentially studied Dpl levels, to evaluate their changes in the time as well as the influence of the disease progression and of therapy, whereas in AML patients treated with aggressive polychemotherapy Dpl levels were sequentially quantified to assess minimal residual disease. Immunocytochemistry, flow cytometry and molecular (real-time quantitative PCR) studies were carried out on bone marrow cells from 64 AML patients at diagnosis, after induction therapy and at relapse, and from 45 MDS patients at diagnosis and during disease progression. Controls were 14 non-hemopathic subjects. Dpl, barely detectable in normal controls, was detected in almost all AML and MDS cases, with median percentages of positive cells of 11.5% (IQR 7-24%), and 17.5% (IQR 11-29%) respectively. In AML no significant relationship was observed between Dpl levels and clinical and laboratory features nor did Dpl levels predict response to therapy. In patients achieving complete remission (22/34) a significant reduction of both transcript and protein levels (p=0.02) were observed. In 10 relapsing patients Dpl was again overexpressed at similar levels to those observed at onset. Also in MDS, Dpl levels were unrelated to clinical and laboratory aspects nor did they predict disease progression. Their behaviour was variable during evolution towards acute leukemia. In pathological samples Dpl was abnormally localized in the cell cytoplasm. This localization was probably dependent on abnormal cellular trafficking because of glycosylation pattern modifications of the protein. In conclusion, our findings confirm the clinical usefulness of Dpl quantification for AML or MDS diagnosis and for the assessment of minimal residual disease. Moreover, they suggest its possible physiopathological role at least in the early phases of cell transformation. Studies are in progress to better understand which factors may contribute to the modulation of PRND activity: identifying the promoter region and critical elements for the activation of the gene may provide new insights into the involvement of Dpl in leukemic transformation.

CO-022

HYPERMETHYLATION OF THE SUPPRESSOR OF CYTOKINE SIGNALING-1 (SOCS-1) AND THE SH2-CONTAINING PHOSPHATASE-1 (SHP-1) GENES THROUGHOUT THE SPECTRUM OF PHILADELPHIA NEGATIVE CHRONIC MYELOPROLIFERATIVE DISORDERS (PH- CMPD)

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Aberrant DNA methylation of promoter CpG dinucleotides is associated with the silencing of many genes in human malignancies, including the negative regulators of cytokine signaling SHP-1 and SOCS-1. SHP-1 plays a critical role in signaling required for appropriate hemopoiesis acting as a direct antagonist of EPO receptor and JAK2 phosphorylation/activation. SHP-1-/- mice display features of myeloproliferation including splenomegaly and expansion of the myeloid compartment. SOCS-1 is induced in response of a subset of cytokines, including EPO and G-CSF and has a crucial function in the negative regulation of JAK2 activation. Philadelphia negative chronic myeloproliferative disorders (Ph- CMPD) are a clinically overlapping group of disorders characterized by a novel somatic point mutation of the JAK2 gene, that occurs within the enzymatically inactive JH2 pseudo-kinase domain, leading to constitutive JAK-STAT activation. Here we tested Ph- CMPD for the involvement of other genes implicated in JAK2 signaling. Forty four Ph- CMPD, including 22 essential thrombocythemia (ET), 12 polycythemia vera (PV), 4 idiopathic myelofibrosis (IMF), 4 chronic myelomonocytic leukemia (CMML) and 2 Ph- chronic myeloid leukemia (Ph- CML), were analysed for SOCS-1 and SHP-1 aberrant methylation by methylation-specific PCR. Cases were also analysed for JAK2V617F mutation by allele specific PCR. For comparison, 10 samples of normal bone marrow hematopoietic cells were also investigated. Methylation of SHP-1 occurred in 4/12 (33.3%) PV and 3/22 (13.6%) ET, while it was absent in IMF (0/4), CMML (0/4) and Ph- CML (0/2). Methylation of SOCS-1 occurred in 4/12 (33.3%) PV, 4/22 (18.2%) ET, and 2/4 (50.0%) IMF, while it was absent in CMML (0/4) and Ph- CML (0/2). All normal bone marrow samples (n=10) scored negative for SHP-1 and SOCS-1 methylation. JAK2V617F mutation was detected in 25/44 (56.8%) Ph- CMPD, including 10/12 (83.3%) PV, 12/22 (54.5%) ET and 3/4 (75.0%) IMF. All CMML and Ph- CML were germline in the JAK2 gene. SHP-1 and SOCS-1 methylation was analysed according to JAK2 mutation status in PV, ET and IMF. In this group of patients, SHP-1 and SOCS-1 methylation occurred in both JAK2 mutated cases (5/25, 20.0% for SHP-1; and 8/25, 32.0% for SOCS-1) and in germline cases (2/13, 15.3% for SHP-1; and 2/13, 15.3% for SOCS-1). By combining the results of SHP-1 and SOCS-1 methylation status, 10/25 (40.0%) JAK2 mutated cases carried SHP-1 and/or SOCS-1 methylation as opposed to 3/13 (23.1%) germline cases. Finally, considering cases methylated for SHP-1 and/or SOCS-1, the vast majority (10/13; 76.9%) also displayed JAK2V617F mutation. This pattern of SHP-1 and SOCS-1 methylation was conserved also when the analysis was restricted to PV, ET and IMF each as a single group and after stratification for JAK2V617F mutation. The implication of these results are threefold. First, inactivation of SHP-1 and SOCS-1 by aberrant methylation is involved in the pathogenesis of Ph- CMPD and is selectively associated with neoplastic hemopiesis. Second, among Ph- CMPD, SHP-1 and SOCS-1 methylation clusters with categories that are also targeted by JAK2V617F mutation, namely PV, ET and IMF, while it appears to be absent in CMML and Ph- CML. The finding of SHP-1 and SOCS-1 methylation further underscores the central role of JAK2-STAT signaling in the pathogenesis of PV, ET and IMF. Third, among PV, ET and IMF cases, SHP-1 and/or SOCS-1 methylation seems to display a correlation with JAK2V617F mutation, suggesting a redundancy of the mechanisms implicated in aberrant JAK2 tyrosine-kinase signalling.

CO-023

SPECIFIC DEGRADATION OF THE AML1-ETO/COREPRESSOR COMPLEX BY A NOVEL ORAL HYDROXAMIC ACID DERIVATIVE

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Alterations in chromatin organization are a common shared mechanism in leukemogenesis. On this basis, many drugs affecting epigenetic modulations have been proposed as therapy of hematopoietic neoplasms, most of the times, unfortunately, without molecular selection of the types of disease more prone to respond to these agents. Nevertheless, hypomethylating agents have shown outstanding efficacy in myelodysplastic syndromes and acute leukemias, whereas histone deacetylase inhibitors (HDACi) seem the most powerful therapeutic tools in T cell cutaneous lymphomas. We think that HDACi could be essential in the treatment of some forms of acute myeloid leukemias, i.e CBF-AMLs. We analysed the effects of a new hydroxamic acid oral derivative, ITF2357 on AML1-ETO positive AML blast cells. We compared both the efficacy of ITF2357 with that of SAHA, and its activity on non CBF-AML blast cells. ITF2357and SAHA were employed at equimolar concentrations (0.1, 0.5, 1, 5 μM) in cell culture. Kasumi-1 and primary AML1ETO pos cells, HL 60 cells and THP-1 cells were cultured

in RPMI medium, 10% FCS, exposed at drugs for 3-6-24-48-72-96 hours. At the end of culture, cell proliferation and apoptosis were evaluated in parallel with histone acetylation. ITF2357 0.1microM was able to block proliferation of CBF-AML cells and induce significative apoptosis (evaluated as Annexin V positivity) after 24 hours, whereas SAHA induced the same rate of apoptosis and cell growth inhibition at 1 microM dose. Non CBF pos AML cells were significantly less sensitive to ITF2357 and SAHA inhibitory effects. When we evaluated histone H3 and H4 acetylation, maximal acetylating activity of ITF2357 was demonstrated in AML1 ETO pos cells after 6 hours of exposure to the drug, was mantained up to 24 hrs, and then was decreasing. ITF2357 0.1microM was able to induce potent acetylation. SAHA induced significantly histone H3 and H4 acetylation with a time schedule similar to the new hydroxamic derivative, but at doses 1 log higher. When we studied at confocal microscope the cellular distribution of the AML1ETO/DNMT/HDAC complex, we could demonstrate, after treatment of Kasumi-1 cells with ITF2357 0.1 microM, a displacement of DNMT1and HDAC1 from nucleous to cytoplasm paralleled by degradation of AML1ETO protein. At the same time, p300 was spotted inside the cell nucleous. The specificity of action of the HDACinhibitor on cells bearing the AML1ETO oncogene was demonstrated Chromatin Immunoprecipitation (ChIP) of acetylated histone H4. Consistently with our evidences at immunofluorescent staining, in Kasumi-1 cells, treatment with ITF2357 restored the transcription of IL-3, whose gene is under direct control of AML1ETO. The pattern of methylation of the promoters of several genes (p15ink, e-cadherin, DAPkinase, IL-3) was analysed, but we could not demonstrate by methylation specific PCR and sequencing any decrease in CpG hypermethylation induced directly by treatment with ITF2357. In conclusion, ITF 2357 is the most potent oral HDACi of the hydroxmic acid family tested so far *in vitro* on AML cells. Its activity reaches a maximum rapidly and effects are stable for 24 hours. ITF2357, but also other HDACinhibitors show a significant specificity for AML1ETO cells in terms of biological effects and of molecular activity on deflecting the stability of the DNA binding of DNMT/HDAC repressor complex. Our observations strongly sugget that therapy with HDACi should be considered only for molecular subtypes of AML in which chromatin organizing protein are involved in the pathogenesis of the disease and thus demonstrated HDACi specific activity may influence the natural history of the leukmeia, most probably as we also demonstrated with the support of hypomethylating agents. ITF2357 is in Phase II clinical trials for myeloma and inflammatory chronic diseases and deserves to be considered as a targeted drug to implement therapy of CBF AMLs.

CO-024

LOSS OF HETEROZYGOSITY IN ACUTE MYELOID LEUKEMIA: EVIDENCE OF FREQUENT CRYPTIC CHROMOSOMAL DELETIONS

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Clonal chromosomal abnormalities are detected by conventional cytogenetic analysis (CCA) in 40-60% of de novo acute myeloid leukemia patients (AML); most of cytogenetic aberrations consist of balanced chromosomal translocations or unbalanced karyotype abnormalities such as gains and/or losses. CCA is a fundamental component of modern leukemia cytogenetics; nevertheless cryptic chromosomal aberrations may be detected in some patients only by molecular genetic analysis. Loss of heterozygosity (LOH) derives from deletion of one allele at a given locus; it has been used to detect genetic imbalance in several neoplasms. LOH technique has a higher resolution power than CCA and FISH because of the ability to detect small (few kb) DNA losses. To determine the frequency of submicroscopic deletions identified by LOH, we performed microsatellite allelotype assay in 74 acute myeloid leukemias (41 female and 33 male; age range: 10-77 years; FAB distribution: 6 M0, 13 M1, 18 M2, 10 M3, 14 M4, 6 M5, 5 sAML and 2 MDS). The same patients were studied also by conventional cytogenetics. We used 35 highly informative microsatellite repeat markers covering eight chromosomal regions known to be frequently involved in AML. In particular we selected polymorphic markers spanning bands 5q23.3-31.2, 6q16-22.3, 7q22.1-32.3, 9p13-21.3, 12p12.2-13.3, 13q13, 17p12-13.3 and 20q11 to investigate the incidence of LOH and its relationship with karyotype. Of the 74 patients, 63 (85%) had an evaluable karyotype (38 altered karyotype and 25 diploid); 42 pts. (57%) showed microsatellite DNA imbalance at the screened loci. Of them, 4 had chromosomal monosomies or deletions in keeping with the microsatellites data, whereas the remaining 38 cases had no cytogenetically detectable abnormalities at the screened bands. Karyotype abnormalities were detected in 24/34 (70%) LOH+ pts. and in 14/29 (48%) LOH- pts. (p=NS). Among 25 pts with diploid kariotypes 10 showed LOH (40%). A total of 2028 loci was successfully screened, and 84 genetic imbalances were observed [69 LOH (82%), 7 genomic amplifications (8.3%), and 8 genetic microsatellite instability (9,5%)]. LOH events were randomly present among all analyzed chromosome bands without any particular distribution, except for a high frequency (n=28) on 7q22-32. Our preliminary data show that LOH is a common event in acute myeloid leukemia even in patients whose karyotype is apparently diploid. Cryptic deletions of wild type allele may cause homozygosity of a mutated allele of a tumor-suppressor gene. Alternatively, LOH may influence gene expression pattern by the loss of siRNA or as a consequence of gene dosage. All of these molecular mechanisms may have important implications in leukemogenesis.

CO-025

DAP-KINASE HYPERMETHYLATION AND APOPTOSIS IN MYELODYSPLASTIC SYNDROMES

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Epigenetic alterations, including aberrant CpG island methylation, in the promoter region of multiple genes, leading to silencing of tumor suppressor genes, have gained increasing recognition as important factors in tumor development and progression in myelodysplastic syndromes. The potential reversibility of epigenetic changes is the rational for the use of demethylating agents, such as decitabine and azacitidine, to treat MDS. Among genes regulated by promoter hypermethylation, death-associated protein kinase (DAP-kinase) is a proapoptotic calcium/calmodulin-regulated serine/threonine kinase that participates in several apoptotic pathways initiated by interferon γ (INF-γ), tumor necrosis factor α (TNF-α), activated Fas, and detachment from extracellular matrix. In our previous work we found DAP-kinase hypermethylation in about 40% MDS (Voso *et al.,* Blood, 2004). We now extended this observation to a well characterized group of 102 patients and studied the functional significance of DAP-kinase expression and apoptosis, also following treatment with demethylating agents. Using methylation-specific PCR (MSP), similarly to our previous report, we found in the present study that 38% of samples had a methylated DAP-kinase (39/102 patients). No significant differences were found when grouping patients according to age, type of MDS, karyotype, previous malignancies, peripheral blood cell counts or IPSS score. We were then interested on the dynamics of DAP-kinase promoter hypermethylation and studied sequential samples from 11 MDS patients during follow-up, in the presence of disease progression (n=8 patients), at a median of 12.4 months (range 1.8-40.6 months) from initial diagnosis. Most patients, 7 of whom with disease progression, showed the same profiles of unmethylated (n=6) or methylated DAP-kinase (2 patients), while only 2 patients gained DAP-kinase hypermethylation, and one patient became unmethylated. Since MDS samples may be heterogeneous for cell content, we freshly isolated $CD34^+$ cells from MDS samples and found that DAP-kinase methylation status was similar in the $CD34^+$ and $CD34^-$ cell fractions. DAP-kinase hypermethylation in was associated to reduced mRNA expression in $CD34^+$ cells, when compared to unmethylated samples. Since DAP-kinase has been shown to be important for apoptosis, we studied the relation between apoptosis in the bone marrow at the time of initial diagnosis and DAP-kinase hypermethylation. Indeed, the proportion of apoptotic cells, studied by fluorocytometry, was higher in total bone marrow MNC and CD34+ cells of patients with unmethylated (n=12), when compared to methylated DAP-kinase (n=8, p=0.068 and p=0.1, for MNC and CD34+ cells, respectively). We then used the HL-60 cell line model to study the effects of treatment with demethylating agents on DAP-kinase function. Indeed MSP showed restoration of the unmethylated state of DAP-kinase after exposure to 1 μM decitabine, associated to re-expression of DAP-kinase transcripts. When looking at cell toxicity and apoptosis, we

found that the combination of 5-AZA and the hystonedeacetylase inhibitor trichostatin A (TSA) led to higher efficacy, than treatment with either inhibitor alone (n= 5 experiments). When compared to controls, this corresponded to a mean 65% HL-60 cell growth inhibition for AZA (p=0.0004), 32% for TSA (p=0.3) and 82% for the AZA/TSA combination (p=0.03, compared to AZA alone). Correspondingly, following 4 days of culture, apoptosis was 2.2±0.3% in the control, 11.3±2.8% in the presence of AZA (p=0.02), 5.7±2.3% in the presence of TSA (p=0.13), while the combination of the two had a synergistic effect, with apoptosis increasing to 41.9±9.8% (p=0.009).These data show that reactivation of DAP-kinase may have an important role in the therapeutic effects due to epigenetic treatment in MDS.

CO-026

MICRORNAS: POSSIBLE ROLE IN THE MOLECULAR AETIOLOGY OF CHRONIC MYELOPROLIFERATIVE DISEASES

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microRNAs (miRNAs) are a large class of tinny regulatory RNAs that act as post-transcriptional modulators of gene expression through base pairing to partially complementary sites in the 3' untranslated regions (3'UTR) of the targeted messenger RNA. This interaction leads to reduced translation of the proteins encoded by these transcripts (natural RNAi). Up to date, more than 300 miRNAs have been cloned in mammals. Although very little is known about the specific miRNAs targeted by miRNAs, several evidences link the expression of certain miRNAs to regulation of hematopoiesis and to leukaemia or lymphoma genesis. The localization of human miR-15a/miR-16 cluster and miR-146 at the chromosomal breakpoint or deletion sites typically found in chronic lymphocytic leukemia (CLL) and multiple myeloma suggested the possible involvement of those miRNAs in the pathogenesis of these disorders. Interestingly, in several human CLL cases, miR-15a and miR-16-1 expression was abolished or diminished. In contrast, the expression of miR-181 is higher in mature B lymphocytes than in mouse bone marrow, suggesting a role of miR-181 for B-cell differentiation; in addition high expression of miR 223 seems to be essential to granulocyte differentiation. We have recently observed by microarray analysis in a model cell line for myeloid differentiation that four miRs (out of 248 screened) change their expression after differentiation and, therefore, they may play a role in this process. Taken together, these data support the idea that miRs can act either activating cell proliferation or directing differentiation. Our working hypothesis is that miRNAs can play a role also in the pathogenesis of chronic myeloproliferative diseases such as Polycytemia Vera (PV) and Essential Thrombocytemia (ET). As it has been shown that Jak2 protein may play a role in both diseases, we made in silico analysis to determine whether Jak2 mRNA can be targeted by miRNAs. We found two candidates, miR 320 and miR 377, as putative regulators of Jak2 translation. Preliminary results obtained in ten patients showed increased expression of miR 320 in this disordes; the level was higher in peripheral blood compared to bone marrow. This higher expression can be due to a more efficient processing of the precursor miR 320 into the active form. We are analyzing the levels of Jak2 protein in the same samples to correlate them it with miR 320 levels. Moreover, we plan to extend our analysis performing miRNAs expression profile, by microarray analysis, on larger cohorts of PV and ET patients.

CO-027

ABERRANT METHYLATION OF ANTI-ANGIOGENIC PROTEINS IN MYELODISPLASTIC SYNDROMES

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Angiogenesis is a complex process involving extensive interactions between cells, extracellular matrix components and soluble factors. Angiogenesis is an essential component of the normal physiology of development, but it contributes also to the pathogenesis of a variety of disease, such as rheumatoid arthritis and benign and malignant neoplasia. The pathways of neo-angiogenesis in myelodisplastic syndromes (MDS) are not clear, but several studies showed that microvessel density in patients with MDS is higher than in controls. Based upon these investigations, small molecules, inhibitors of angiogenic cytokines, such as thalidomide and its analogue lenalidomide, have emerged as a promising class of therapeutics for MDS. Cadherin is a superfamily of calcium-mediated membrane glycoproteins. E-cadherin (CDH1) is the prototype of the class, linking to catenins to form the cytoskeleton. CDH1 gene has been identified as a metastasis suppressor gene. Thrombospondin is a family of glycosylated extracellular matrix proteins, that modulate interactions between cells and the cellular environment, regulate cell adhesion and are typically expressed during tissue formative processes. Thrombospondin-1 (TBSP-1) is a very effective inhibitor of angiogenesis. Cyclooxygenase-2 (COX-2) is a pro-angiogenic protein, not expressed by most of normal tissues, but induced by inflammatory and mitogenic stimuli. Our purpose was to determine whether inhibition of CDH1, TBSP-1 and COX-2 due to promoter methylation plays a role in MDS. Using a methylation-specific PCR, we studied promoter hypermethylation of CDH1, TBSP-1 and COX-2 in 91 patients with MDS (34 RA, 9 RARS, 30 RAEB, 15 CMML/MP-MDS, 3 5q- syndromes). Promoter hypermethylation was related to patients' characteristics and survival. Hypermethylation of CDH1 was present in 31% (28/91) of MDS samples. It was significantly more frequent in thrombocytopenic patients (platelets count < 50000/μL), as compared to patients with platelets count > 50000/μL (12/19, 63%, versus 15/70, 21%, p=0.001, OR=6.3, 95% C.I.2.1-18.8). No association was found with other patient's characteristics, including age, sex, karyotype, IPSS, WBC count, blast percentage in the peripheral blood and overall survival. Hypermethylation of TBSP-1 was present in 6.5% (6/91) of MDS samples. It was frequent in patients with high IPSS, when compared to patients with low-intermediate IPSS (3/5, 60%, versus 2/61, 3%, p=0.03). Moreover, hypermethylation of TBSP-1 was a frequent event in patients with blasts in peripheral blood (2/11, 18%, versus 3/77, 4%, p=0.1) and was associated to reduced overall survival (p=0.01). We did not find COX-2 hypermethylation in our MDS patients. Hypermethylation of anti-angiogenic proteins could be important in the pathogenesis of MDS. In particular, reduced E-Cadherin expression could be one of the pathogenetic mechanisms of the impaired thrombocytopoiesis observed in MDS. Accordingly, it has been shown in a mouse model that inhibition of VE-Cadherin results into a magakaryocytopoiesis block and reduced platelet counts (Avecilla *et al,* Nat med 2004). Furtrhermore, since thrombospondin is important for interactions between cells and environment, inhibition of adhesion due to thrombospondin hypermethylation in MDS could lead to a more aggressive phenotype.

Chronic Myeloid Leukemia

CO-028

BINDING MODE OF THE NOVEL DUAL SRC AND ABL INHIBITOR SKI-606 TO THE BCR-ABL KINASE AS PREDICTED BY MOLECULAR DOCKING STUDIES

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Background. SKI-606 is a novel 4-anilino-3-quinolinecarbonitrile Src and Abl kinase inhibitor. SKI-606 has been shown to be a potent antiproliferative and proapoptotic agent when tested on Bcr-Abl-positive cell lines. The remarkable efficacy of SKI-606 against chronic myeloid leukemia (CML) cells in culture was mirrored by its activity *in vivo* against CML xenografts: K562 tumors regressed in nude mice when SKI-606 was administered per os once daily over a 5-day period. The crystal structure of the Bcr-Abl kinase domain in complex with SKI-606 has not yet been determined and the mode of binding of this inhibitor is therefore unknown. Moreover, there are currently no published data on the ability of SKI-606 to bind and efficiently inhibit the Bcr-Abl mutants known to confer resistance to imatinib. *Aims.* In this study, we used a molecular docking approach to a) determine SKI-606 binding mode to the wild-type (wt) form of the Bcr-Abl kinase; b) hypothesize SKI-606 binding mode to the more frequent, clinically relevant Bcr-Abl mutants known not to be inhibited by imatinib; c) predict which novel mutant forms might emerge and interfere with SKI-606 binding. *Methods.* Modelling of the human Abl kinase was performed with the program Modeller v7.7 (http://salilab.org/modeller) adopting the highly related Mus musculus Abl homologue as a template structure (PDB: 1OPJ, 0.175nm resolution). Chemsketch (http://www.acdlabs.com) was used to build a three-dimensional model of SKI-606. Flexible docking of the ligand to the protein was performed with Autodock v3.0 (http://www.scripps.edu/mb/olson). *Results*. We first docked SKI-606 on Bcr-Abl with the activation loop in the active (open) and inactive (closed) conformation (the latter is the one to which imatinib binds). According to our results, the interaction between SKI-606 and Bcr-Abl seems to be more stable when the activation loop is in the inactive conformation. The consequent structural study of SKI-606 modeled into wt-Bcr-Abl ATP binding site highlighted the variant residues located within a spherical environment of 0.5nm centered on SKI-606: Y253, T315 and F359 (residues numbered according to ABL exon Ia splice variant). The binding of SKI-606 to the eight Bcr-Abl mutants which are most frequently implicated in clinical resistance to imatinib mesylate was also studied: G250E, Y253H, E255K, T315I, M351T, F359V, H396R. Our results indicated that SKI-606 retains the ability of efficiently binding all the above mentioned Bcr-Abl variants with the exception of the T315I mutant. Finally, we identified six potential residues around SKI-606 that, if mutated, could potentially be able to interfere with the SKI-606/Bcr-Abl interaction: a) the charged residues K271, D381 and H361; b) the hydrophobic/aliphatic residues V299, A380 and M318. *Conclusions.* Pre-clinical data suggest that SKI-606 is a promising second-generation kinase inhibitor with potent antiproliferative and proapoptotic effects on CML cells. Our docking experiments indicate that SKI-606 may prove effective in imatinib-resistant patients since it is expected to retain the ability to bind several Bcr-Abl mutant forms. A phase I trial is about to start in CML and Philadelphia-positive acute lymphoblastic leukemia.

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CO-029

IMATINIB 800 MG IN INTERMEDIATE SOKAL RISK CML PATIENTS IN EARLY CHRONIC PHASE: RESULTS OF A PHASE II TRIAL OF THE GIMEMA CML WP

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Background. Imatinib standard dose (400 mg) gives impressive results in early chronic phase chronic myeloid leukemia (CML). Results, stratified by Sokal risk, are inferior in intermediate and high risk with respect to low risk. In intermediate Sokal risk patients, the IRIS trial (T Hughes et al, NEJM 349:15, 2003) reported within 12 months a complete cytogenetic response (CCgR) rate of 67% and a major molecular response (MMR) rate of 45%. Phase I and II trials of imatinib have clearly shown a dose response effect. Kantarjian et al (Blood 103, 2004) reported higher response rates with imatinib high dose (800 mg) in 114 early chronic phase patients treated at the MD Anderson Hospital. The CCgR was 90% and MMR 60% within 12 months of therapy. *Aims.* The GIMEMA CML WP opened in January, 2004 a phase II, multicentric prospective study (serial n. CML/021) devoted to investigate the effects of imatinib high dose (800 mg) in intermediate Sokal risk patients. *Methods.* Clinical and anagraphical data were collected through a web-based system. Responses were evaluated at fixed time-points during treatment: hematologic, continuosly; cytogenetic, at 6 and 12 months (local labs); molecular, at 3, 6 and 12 months. Peripheral blood samples for quantitative molecular analysis (RT-Q-PCR, Bcr-Abl/Abl x 100 - Taqman) were centralized in Bologna at 3, 6 and 12 months. *Patients.* Between January 1, 2004 and May 25, 2005 25 italian centers enrolled 82 patients (80 evaluable, 46 males and 34 females). Median age was 56 yrs (range 26-79). 80 patients are evaluable for response at 3 months, 76 at 6 months and 63 at 12 months. The median observation time is 12 months. *Results.* At 3 and 6 months, 83% and 97% of the patients reached a stable complete hematologic response, respectively. At 6 months, 86% of the evaluable cases obtained a CCgR (100% Ph-neg). A MMR defined as a Bcr-Abl/Abl × 100 ratio < 0.1%, was shown in 48% of CCgR patients. At 12 months, the CCgR rate was 88% and the MMR rate in CCgR patients was 47%. The cumulative incidence of CCgR was 92%. 2 patients progressed to accelerated/blastic phase and 1 patient definitively suspended imatinib to be enrolled in a phase II study of a new tyrosine kinase inhibitor (no cytogenetic response after 6 months). 55%, 53% and 52% of the pts received 100% of the scheduled dose at 3, 6 and 12 months. *Summary and Conclusions.* The preliminary results of our trial suggest that imatinib 800 mg is highly effective for intermediate Sokal risk CML in early chronic phase, being the cytogenetic response rates superior to 400 mg (IRIS trial, same risk category) and in the range of the MD Anderson results.

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CO-030

DERIVATIVE CHROMOSOME 9 DELETIONS IN CHRONIC MYELOID LEUKEMIA: A MOLECULAR CYTOGENETIC STUDY ON 334 CASES AT DIAGNOSIS

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Chronic myeloid leukaemia (CML) is characterized by a reciprocal translocation t(9;22)(q34;q11) that generates a BCR-ABL fusion gene on the derivative 22 called the Philadelphia (Ph) chromosome. Large deletions adjacent to the t(9;22) breakpoint on the derivative 9 chromosome have now been found, which result in genomic loss at both sides of the translocation breakpoint. We report an update of our FISH study on CML cases bearing deletions on der(9) chromosome. FISH analysis with BAC/PAC clones specific for ABL1 and BCR genes (as previously reported) was performed on bone marrow cells of 334 CML patients at diagnosis. A set of BAC/PAC probes, belonging to chromosomes 9, 22 and to the third chromsome involved in variant rearrangements, was selected according to the University of Santa Cruz (UCSC) database and employed in FISH experiments. UCSC database was also queried for genes with known function mapping inside deleted regions. We have detected der(9) deletions in 60 (18%) CML cases. Deletions of chromosome 9 sequences on the der(9) were found in 50 (83%) cases; they were present in all Ph+ metaphases and ranged from 350 Kb to 41.6 Mb. A tumor suppressor gene (TSG) called prostaglandin E synthase (PTGES) was lost in 39 (78%) cases. Chromosome 22 deletions on der(9) were found in 52 (87%) of the analysed cases; the deleted chromosome 22 sequences were shorter than the deleted chromosome 9 sequences (rang-

ing from 400 Kb to 12.7 Mb). Two TSGs mapping inside the deleted sequences of chromosome 22, SMARCB1 and GSTT1, were found deleted in 37 (71%) cases. Thirty-two (10%) CML patients showed a variant 9/22 rearrangement. Thirteen (41%) of them were deleted on der(9) chromosome; moreover, in 9 out of 13 cases genomic loss were detected on the third chromosome involved in the variant t(9;22) translocation. The observation that deletions on der(9) are associated with the loss of TSGs suggests their possible involvement in the CML outcome, mediated by a haplo-insufficiency mechanism. Future work will aim to clarify whether in CML patients bearing TSGs loss and treated with Imatinib, the duration of the response to treatment is comparable to that of patients without deletions on der(9).

CO-031

ACETYLOME AND PHOSPHOPROTEOME MODIFICATIONS OF IMATINIB RESISTANT CML CELLS AFTER SHORT CHAIN FATTY ACID HISTONE DEACETYLASE INHIBITOR TREATMENT

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CML patients may become irresponsive to Imatinib because of the development of resistance caused by amplification of the BCR-ABL genomic locus or by point mutations within the kinase domain of BCR-ABL, resulting in a block of drug binding. Innovative dual SRC/ABL kinase inhibitors with higher power against native and imatinib-resistant mutants of BCR-ABL give remarkable therapeutic benefits, but at least one mutation remains resistant to any kinase inhibitor (T315I). Given these evidences, the investigation of alternative therapeutic agents effective in CML still remains a subject of primary interest. We analysed whether HDACIs short chain fatty acids (SCFAs) valproic acid and butyrates showed synergistic effects with imatinib as demonstrated for hydroxamic acids. The activity of SCFAs in promoting acetylation of non-histone proteins is not well characterized, we thus compared the acetylated proteome of CML cells treated and not treated with HDACIs, alone and in combination with imatinib, by immunoproteomic techniques and monitored the changes in global phosphorylated proteins after treatment the same drugs. The human CML cell lines K562, KBM, LAMA-84 S (Imatinib sensitive) and LAMA-84 R (Imatinib resistant), and primary imatinib-resistant CML-BC cells were grown in the presence of valproic acid at the escalating doses 0.2 mM to 2mM or in the presence butyric acid derivative D1 (0.2-1 mM) for 24 and 48 hrs. Apoptosis was monitored by annexin V test and propidium iodide uptake. The copy number of bcr-abl mRNA was measured by real time PCR. Bcr-Abl and HSP 90 protein expression was determined by western blot with specific antibodies. LAMA 84-S and –R total cell proteins were separated by 2D electrophoresis (pH 3-11). We used a anti-pan-acetylated and anti-phosphotyrosine antibody for 2D western blots, followed by matching with 2D gel and MALDI-TOF mass spectrometry for protein identification. Apoptosis resulted to be induced in a time and dose dependent way by VPA and D1. Imatinib was synergistic with both HDACIs in inducing apoptosis and cell proliferation arrest (MTT-assay). VPA and D1 were able to induce (48 hrs of incubation) a significant decrease in the number of copies of BCR-ABL determined by real time-PCR both in sensitive and in resistant cells. Moreover, a concomitant significant decrease in BCRr-ABL protein expression was observed by western blots of total cell lysates from CML cells. The lower expression level of protein kinase could be due to the synergy with imatinib, but also to the reversal of resistance in mutated Bcr-Abl CML cells and is consistent with the previous results. We also analysed the expression of Hsp-90, known to be a protein chaperone of Bcr-Abl, and found its expression level not substantially modified, but the protein resulted to be hyperacetylated by the treatment with both HDACIs. Twenty two proteins differentially acetylated were identified. At least two chaperone proteins were identified as target of acetylation after VPA and D1 treatment of CML cells, other targets were proteins involved in the synthesis and stability of RNA. Sixteen proteins differentially phosphorylated were identified. For 13 of these proteins the phosphorylation level was not significantly affected by HDACIs in resistant cells, while the combination of both Imatinib and HDACIs produced a considerable decrease of phosphorylation in both sensitive and resistant cell lines. This category includes: HSP90, HSP70, HOP1 and nucleophosmin. Even if short chain fatty acids are not the most powerful HDACIs, they have been used successfully in clinical trials. Our analysis show significant evidences of their effects on CML cells in terms of induction of apoptosys and arrest of CML cell proliferation. Further effects on Bcr-ABl expression and modifications on both acetylome and phosphoproteome were proved in synergistic manner with imatinib. The results of this study bring to characterize the proteome modifications given by HDACIs and may help to understand the molecular effects of different HDACIs on CML cells in order to improve their use as single drugs or in combination with imatinib or new SRC/ABL inhibitors.

CO-032

T315I AND F317L ARE THE ONLY BCR-ABL MUTANTS WHICH SEEM TO CONFER RESISTANCE TO DASATINIB (BMS-354825) TREATMENT IN PHILADELPHIA-POSITIVE LEUKEMIA PATIENTS RESISTANT TO OR INTOLERANT OF PREVIOUS IMATINIB TREATMENT

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Resistance to the Bcr-Abl tyrosine kinase inhibitor imatinib mesylate occurs in a small but significant proportion of patients (pts) with chronic phase (CP) chronic myeloid leukemia (CML) and in most of the patients with advanced-phase CML and with Philadelphia-positive (Ph+) acute lymphoblastic leukemia (ALL). Resistance is often triggered by the selection of mutations in the Abl kinase domain (KD) which abrogate imatinib binding without impairing kinase activity. Dasatinib (BMS-354825) is a novel dual Src and Abl tyrosine kinase inhibitor which showed greater potency in BCR-ABL inhibition, less stringency in binding, and high effectiveness against several ABL KD mutants in pre-clinical studies. Since February 2005, 38 pts (14 pts with CP CML and 24 pts with advanced-phase CML or Ph+ ALL) who were resistant to or intolerant of imatinib have been treated with dasatinib at our center. Median follow-up is 9 (1-14) months. Prior to therapy and every month thereafter, bone marrow and peripheral blood samples were collected and analyzed for the presence of ABL KD mutations by denaturing-high performance liquid chromatography (D-HPLC) and sequencing. Mutation analysis before the onset of dasatinib treatment revealed the presence of 26 mutations in 24/38 (63%) pts (6/14 [43%] pts with CP CML and 18/24 [75%] pts with advanced phase CML or Ph+ ALL). Mutations were M244V (2 pts), G250E (3 pts), Y253H (5 pts), E255K (3 pts), D276G (1 pt), T315I (4 pts), F317L (1 pt), M351T (3 pts), L387M (2 pts), H396R (2 pts). Mutation analysis at follow-up revealed the disappearance of specific mutants both in CP CML and in advanced phase CML/Ph+ ALL pts (n=13). Bcr-Abl mutants which were eliminated by dasatinib were M244V, Y253H, D276V, M351T and H396R. On the other hand, novel mutant clones emerged in advanced phase/Ph+ ALL pts (n=7). Bcr-Abl mutants which were selected by dasatinib were T315I and F317L. So far, 13 pts have experienced disease progression, which was invariably associated with the presence or the outgrowth of T315I- or F317L-positive clones. In our experience, dasatinib treatment in CML and Ph+ ALL pts resistant to imatinib proved effective against several Bcr-Abl mutants. The only Bcr-Abl mutants which turned out to confer resistance to dasatinib were T315I and F317L. Threonine 315, the so-called "gatekeeper" residue, sterically controls the accessibility of the active site to the inhibitor. The introduction of a bulkier and more hydrophobic isoleucine side chain into the gatekeeper position creates a steric hindrance which interferes with binding of imatinib as well as of all the other second-generation inhibitors in clinical development. Accordingly, preclinical studies confirmed that the T315I was highly resistant to dasatinib (fold increase in cellular IC50 with respect to wild-type Bcr-Abl, >200). Co-crystal structure of dasatinib in complex with Bcr-Abl has shown that also phenilalanine 317 is a critical contact point. Accordingly, preclinical studies showed that, if we exclude T315I, the F317L mutant displays the highest cellular IC50 among all BCR-ABL mutants. Therefore, F317L might be another problematic mutant in pts treated with dasatinib.

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CO-033

ABSENCE OF SPRED1, A NEGATIVE REGULATOR OF TYROSINE KINASE ACTIVITY, IN CHRONIC MYELOID LEUKAEMIA PATIENTS

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Background. Spred1 proteins are inducible inhibitors of signalling induced by receptor tyrosine kinases. They are implicated in negative feedback interactions that regulate intracellular pathways. The repressive function of Spred proteins targets several TK receptors so resulting in a variety of biological effects. Spred proteins, after growth factor stimulation, translocate to the plasma membrane, become tyrosine phosphorilated and interact with components of the Ras/MAPK and Ras/Raf/Erk pathways. *Aim.* The aim of this study was to assess whether the activation of Bcr-Abl pathway leading to the disruption of many biological processes, could be supported by a defective signalling inhibition. Methods: Using a Real Time PCR we studied the expression level of Spred1 in 80 samples collected from CML patients at diagnosis (15 PB and 65 BM), and 9 BM samples from patients in blastic phase (BC). Furthermore, 12 CP patients were evaluated also at the time of the achievement of complete cytogenetic remission. Finally, 36 normal controls (20 PB and 16 BM) were studied. The protein level was analyzed by western blot and immunofluorescence assay. Sequence analysis of the coding and promoter regions was performed. In order to establish the effects induced of the absence of Spred1 on proliferation, we transfected K562 cells with Spred1 plasmid. After transfection colony growth was evaluated in semisolid medium, the proliferation rate was estimated by MTT assay and by the incorporation of 3H timidine. Results: We found that Spred1 transcript amount is significant reduced in CP CML samples (mean value of 2-ΔΔCt =0,02; range 0,1-0,0002) when compare to normal controls (mean 2,4) with a p value of 0,000002. This difference is even more sound in BC CML cells where Spred1 transcript is 4 logs lower compared to normal controls (2-ΔΔCt =0,0003 p=0,0000001). The expression levels significantly increased after reaching the cytogenetic remission (mean value of 2-ΔΔCt = 0,9; p=0,0007 compared to diagnosis) reaching values similar to normal controls (p=0,09). Western blot demonstrated the reduction or the absence of Spred1 protein in CML cells in CP and BC. By contrast, the protein reappeared after the achievement of cytogenetic remission. Sequence analysis allowed to exclude the presence of mutations in Spred1 coding and promoter regions. In order to better understand the mechanism leading to the abrogation of Spred1 we analyzed the factors responsible for Spred1 transcription. We demonstrated that the transcription factor WT1 binds to and activates the promoter region of Spred1. Moreover, we demonstrated a defective transcription activity of WT1 in CML patients due to the absence of one of the isoforms responsible for transcription. K562 cells transfected with Spred1 (K562+) showed a 55% reduction of the proliferation rate compared to untransfected K562 cells (K562-) Moreover a significant reduction of colony growth was observed in K562+ when compared to K562- (mean value of 25±7 vs 180±12). *Conclusions.* This study clearly demonstrates that the absence of Spred1 protein, a physiological inhibitor of RTK mediated signalling, is a common finding in CML cells and this may support the abnormal proliferation in Bcr-Abl positive cells.

CO-034

HIGH FREQUENCY OF PRE-EXISTING BCR-ABL GENE ATP-BINDING DOMAIN MUTATIONS ASSOCIATED WITH NATURAL GLEEVEC RESISTANCE IN CHRONIC MYELOID LEUKEMIA (CML) PATIENTS

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Imatinib monotherapy is gold standard for CML patients. It is the first targeted therapy for cancers. Although very successfull at all CML stages, imatinib resistance (acquired and natural) has been reported in most of the CML patients, making Imatinib resistance as one of the most emerging issues of pharmacogenomics.Point mutations in ATP-binding domain of BCR-ABL fusion gene has been detected in resistant patients which disturb the binding of Imatinib to its target bcr-abl oncoprotein, leading to resistance. Studies show that in some cases, mutations pre-exist the therapy and lead to natural Imatinib resistance. Detection of pre-existing mutations can help in deciding the potential responders and poor responders of the treatment and can help in adjusting the treatment accordingly, thus leading to personalized medication. In this study, pre-existing BCR-ABL ATP domain mutations were detected in CML patients who developed Imatinib resistance, later on i.e. on initiation of the therapy. ASO-PCR was employed to detect point mutations in CML patients prior to Imatinib therapy. Upon initiation of therapy, follow-up studies were carried out to know the Imatinib resistance.A total 52 patients were studied for three mutations. Mixed mutations were detected in 18 (36%), mutation T1052 in 16 (31%), mutation T932C in 8 (15%) and mutation C944T in 7 (11%) patients. All patients showed resistance to Imatinib after therapy initiation.This research report shows that mutations in BCR-ABL ATP-binding domain exist prior to therapy and mutant clones probably proliferate after therapy initiation, leading to natural Imatinib resistance.These findings of all great clinical importance in managing resistance to imatinib and other such drugs and open ways for personalized medication. It will further lead towards understanding molecular mechamisms of resistance to molecularly targeted cancer therapies and to develop the strategies not only to overcome this resistance but also to design more effective drugs.

Chronic Myeloproliferative Disorders

CO-035

IMPACT OF ABL KINASE DOMAIN (KD) MUTATIONS IN DIFFERENT SUBSETS OF IMATINIB (IM)-RESISTANT PHILADELPHIA (PH)-POSITIVE PATIENTS (PTS) – BY THE GIMEMA-CML WORKING PARTY

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Using denaturing-high performance liquid chromatography and sequencing, we screened for ABL KD mutations 370 IM-resistant Ph+ pts. Median time between diagnosis and IM start at 400-600 mg/d was 40 (0-160) months. Median duration of IM was 32 (0-160) months. Evaluable pts were 297/370 (80%). At the time of analysis, 198/297 (66%) pts were in chronic phase (CP)(44 previously untreated, 154 post-IFN failure), 21/297 (7%) pts were in accelerated phase (AP), 32/297 (11%) pts were in myeloid blast crisis (myBC), 46/297 (15%) were in lymphoid BC (lyBC) or had Ph+ acute lymphoblastic leukemia (ALL). One hundred and fifty-two pts had primary resistance to IM, 145 had acquired resistance. Mutations were found in 127/297 (43%) pts. In 8 patients (2 Ph+ ALL, 2myBC, 2 lyBC, 1 AP and 1 CP post-IFN failure) multiple mutations simultaneously occurred. Mutations mapped to 17 codons, the most frequent ones being E255K/V (21 patients, 17%), Y253F/H (17 patients, 13%), T315I (15 patients, 12%), M351T (14 patients, 11%), F359V/I (14 patients, 11%), M244V (13 patients, 10%), G250E (13 patients, 10%). Four novel amino acid substitutions (F311I; M351V; E355D; F359I) and three novel mutated codons (T277A; E281K; P296H) were detected. Mutations were found in 54/198 (27%) patients in first CP (6/44 (14%) treated with IM frontline, 48/154 (31%) treated with IM after α-IFN failure), 11/21 (52%) AP patients, 24/32 (75%) myBC patients and 38/46 (83%) lyBC/Ph+ ALL patients (CP vs. AP, p=.02; AP vs. BC, p=.02; CP vs. BC, p<.0001). When we examined position and relative frequency of mutations by disease phase, we noticed a trend towards the preferential association of P-loop and T315I mutations and advanced stages of disease. Mutations were associated in 45/152 (30%) patients with primary resistance (8/18 hematologic and 37/134 cytogenetic) and in 82/145 (57%) patients with acquired resistance (12/52 patients who lost CCR, 18/33 patients who lost HR, 52/60 patients who progressed to AP/BC) (primary vs. acquired, p<.0001). Primary or acquired resistance did not seem to significantly differ in terms of type or relative frequency of mutations responsible for, but 40/52 mutated patients who had progressed to AP/BC harbored P-loop or T315I mutations. We conclude that: a) mutations at seven hotspots account for 85% of imatinib-resistant cases; b) mutation screening is important both in case of imatinib failure and in case of loss of response at whichever level; c) advanced-phase CML and Ph+ ALL pts are to be considered a high-risk group, for whom particular attention is strongly suggested; d) the presence of P-loop or T315I mutations should prompt an immediate reassessment of the therapeutic strategy.

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CO-036

COMPARATIVE PROTEOMIC ANALYSIS OF CHRONIC MYELOGENOUS LEUKEMIA CELLS: INSIDE THE MECHANISM OF IMATINIB RESISTANCE

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Development of imatinib resistance represents a critical factor for the therapy of chronic myelogenous leukaemia (CML). Resistance is mainly due to mutations in the abl kinase domain, and to overexpression of Bcr/abl protein, provoked by amplification of the genomic locus. We undertook a comparative proteomic approach of human chronic myeloid leukemia cells Imatinib sensitive and Imatinib resistant, to dissect the molecular mechanism of resistance. In fact, the characterisation of biochemical pathways involved with and connected to Bcr/abl could be extremely useful in identifying new therapeutic targets to bypass resistance to the kinase inhibitor. Total cell protein LAMA 84-S and LAMA 84-R extracts were separated by two dimensional electrophoresis (2DE), and gel images were compared by adequate software in order to establish characteristic protein signatures typical of Imatinib sensitive and Imatinib resistant cells. Matrix assisted Laser Desorption Ionisation-Time of Flight Mass spectrometry (MALDI-TOF MS) analysis allowed the identification of 45 differentially expressed proteins We categorized these proteins into five main functional classes: i) Chaperones and Heat shock proteins ii) Nucleic acid interacting proteins (binding/synthesis/stability), iii) structural proteins, iv) cell signalling and v) metabolic enzymes. i) Heat shock proteins HSP60 and HSP70isoform 1 and 2, valosin containing protein (VCP) known to bind the HSP90-interacting Bcr-Abl complex, resulted to be significantly over expressed in LAMA 84-R cells, indicating a possible involvement of several of these chaperone proteins in the mechanism of Imatinib resistance, via a possible block of bcr/abl proteosome degradation. ii) A relevant number of proteins interacting with DNA and RNA (hnRNPF, hnRNPH1, hnRNPK and eIF3) were found to be more abundant or even expressed only in imatinib resistant cells. iii) Structural proteins: vimentin, α tubulin, γ actin were instead significantly more expressed in imatinib sensitive cells. The identified proteins involved in cell signalling and in metabolic pathways (classes iv and v) resulted differentially expressed in LAMA 84-S and LAMA 84-R, but without a clear *signature*. Bcr/abl and FLT3 are client of chaperon protein HSP90 and it has been shown that HSP90 inhibitors are active in blocking CML cell proliferation. HSP70 is involved in inhibition of apoptosis. Thus the overexpression of these class of proteins seems to be directly responsible for the stability, maintenance and function of Bcr/abl, akt and other tyrosine kinase substrate of bcr/abl. This is fundamental for CML cells, basing imatinib resistance on constitutionally overexpression of bcr/abl. A similar pivotal role in the maintenance of the resistant phenotype may be attributed to RNA stabilizing proteins, like hnRNPF, hnRNPH1, hnRNPK.

CO-037

IMATINIB AND AGING IN CHRONIC MYELOID LEUKAEMIA IN EARLY CHRONIC PHASE: RESULTS OF A SUB-ANALYSIS WITHIN 3 TRIALS OF THE GIMEMA CML WORKING PARTY

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Background. Older age constitutes a poor prognostic variable in Ph+ CML patients, across treatment modalities. Age is included and acts as negative factor in the staging systems most employed (Sokal and Euro). Older patients have been generally excluded from most of the trials employing interferon, due to its toxic effects. Few studies investigated the effect of imatinib in older patients: Cortes *et al,* (Cancer 98, 2003), based on a single center casistic (187 early CP patients overall, 49/187 older than 60 yrs), treated at doses between 400 and 800 mg, showed no difference in response and outcome, observation which needs to be confirmed. *Aims.* To investigate the effects of age on response and compliance to imatinib. *Methods.* A sub-analysis within 3 simultaneously running trials of the GIMEMA (Gruppo Italiano Malattie Ematologiche dell'Adulto) CML WP (n.CML/021, phase II - ima 800 in intermediate Sokal risk; CML/022, phase III- ima 400 vs 800 mg in high Sokal risk, n. CML/023, observational - ima 400 mg) have been performed. Overall, 404 patients have been enrolled (January, 2004- November, 2005): at enrolment 85/404 (21%) were > 65 yrs (median age 71, range 65-85) and 319/404 (79%) < 65 yrs (median age 46, range 18-64). Sokal risk distribution was different between the 2 groups: low Sokal risk cases were 15% in older cohort vs 54% in younger cohort (p<0,01). 21% of older and 22% of younger pts received high dose (800 mg) of imatinib frontline. Timing of response evaluation: hematologic, continuously; cytogenetic, at 6 and 12 months; molecular, at 3, 6 and 12 months. PB samples for quantitative analysis (RT-Q-PCR, Bcr-Abl/Abl × 100 - Taqman) were centralized in Bologna at 3, 6 and 12 months. *Results.* The numbers (%)

of evaluable cases (older/younger) at 3,6 and 12 months were: 85/319 (100%/100%), 59/251 (69%/79%) and 27/141 (32%/44%). At 3 months, both groups achieved a 93% complete hematologic response (CHR) rate. At 6 months, the complete cytogenetic response (CCgR) rates were (older/younger) 66%/80% (p=0,39). The major molecular response (MMR, defined as a Bcr-Abl/Abl × 100 ratio < 0.1%) rates (CCgR only) were 67%/49% (p=0,06). At 12 months, CCgR rates were 81%/88% (p=0,67) and MMR 50%/60% (p=0,04). With a median observation time of 6 months, 1 pt (1%) of older cohort and 4 (1%) of younger cohort progressed to accelerated/blastic phase. *Summary and Conclusions.* This subanalysis was generated from 3 trials with different aims and dosages of imatinib. The observation period is still short. However, it is noteworthy that, notwithstanding a worsen risk distribution of older cases (15% low risk vs 54% for younger), results at 6 and 12 months are comparable. The only significative difference was demonstrated for MMR at 12 months. Consequently, we may foresee that the long-term survival and progression free survival will not differ between the 2 groups.

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CO-038

COMPARISON OF CYTOGENETICS AND INTERPHASE FLUORESCENT *IN SITU* HYBRIDIZATION IN NEWLY DIAGNOSED PH+ CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH IMATINIB MESYLATE. A STUDY BY THE GIMEMA WORKING PARTY ON CHRONIC MYELOID LEUKEMIA

Marzocchi G, Luatti S, Montanari E, Gamberini C, Buontempo F, Baldazzi C, Mancini M, Specchia G, Abruzzese E, Rege Cambrin G, Cuneo A, Zaccaria A, Kerim S, Giussani U, Bernasconi P, Mecucci C, Grimoldi MG, Discepoli G, Zanatta L, Gozzetti A, Palka G, Modaferri B, Amabile M, Castagnetti F, Palandri F, Bosi C, Martinelli G, Rosti G, Baccarani M, Testoni N on Behalf Of Gwp On Cml

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The aims of this first prospective multicentric study were to evaluate the role of cytogenetic pattern and to analyse the correlation between conventional (CC) and molecular cytogenetic response in newly diagnosed chronic myelogenous leukemia (CML) patients in chronic phase (CP) treated with Imatinib Mesylate. Karyotype and interphase fluorescent in situ hybridization (FISH) analyses were performed in 520 enrolled patients between April 2004 and April 2006 by the GIMEMA CML Working Party (WP) in three multicentric studies. Local investigator laboratories (26 labs) and WP reference labs (12 labs) performed both analyses. Cytogenetic examinations was performed at diagnosis, after 3 (only in one study), 6 and 12 months of treatment. At the baseline, 508 patients have been studied and 412 (81%) were valuable for both analyses (CC and FISH). Additional abnormalities in Ph+ clone have been observed in 20 patients (5%). Moreover, 25 (6%) cases showed variant Ph translocations and in 55 (13%) patients the derivative of chromosome 9 was deleted. Their role in the response to treatment and in the follow-up will be investigated. As yet, cytogenetic response (CR) was evaluated in 614 samples and 389 cases were valuable (63%) within 1 year of Imatinib. A strong correlation between the CC and FISH tests was observed (r=0,91; p=0,0082). In 311 (80%) evaluations complete CR (CCR) was established with more than 20 metaphases in 217 cases, meanwhile in 94 CCR cases the number of examined metaphases was lower. In the first group, 177/217 (82%) samples showed absence of bcr/abl rearrangement in FISH, meanwhile 33/217 (15%) carried a low rate of positive cells (1-5%) and the last 7 (3%) showed an higher rate of positive cells (5-27%). In the latter group, 67/94 (71%) didn't show rearrangement in FISH, in 20/94 (21%) the amount of Ph+ cells ranged from 1 to 5% and in the last 7 (8%) was higher (5-20%). Moreover 43/389 (11%) evaluations showed partial CR (PCR). In this group, 37 (86%) showed retention of persisting Ph+ cells ranging from 3 to 30% in CC study and from 1 to 33% in FISH analysis. Moreover we found 5/43 (12%) evaluations of PCR established in more than 20 metaphases in which levels of FISH positive were below to 1%. The last PCR case showed 2% of Ph+ metaphases and 53% of Ph+ cells in FISH: in this case the cytogenetic evaluation was established with 10 metaphases. On the basis of this series of patients, we can suggest there was a good correlation between cytogenetic and FISH tests in terms of the kinetics of disappearance of the bcr/abl rearrangement. FISH is a reliable method to reveal submicroscopic deletions and to monitor the size of the Ph + clone in treated CML patients. However, a good CC analysis remains an excellent approach to the evaluation of response to Imatinib. Moreover it can detect the emergence of every chromosomal abnormalities in Ph positive or negative clone.

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CO-039

MOLECULAR RESPONSE TO IMATINIB IN EARLY CHRONIC PHASE VERSUS LATE CHRONIC PHASE CML PATIENTS IN COMPLETE CYTOGENETIC RESPONSE: A COMPARISON AT 24 MONTHS OF 2 CLINICAL TRIALS OF THE GIMEMA-CML WP

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Background. The introduction of Imatinib (IM) has changed the current approach to the management of chronic myeloid leukaemia (CML). It is currently unclear whether patients (pts) treated with IM first-line treatment have a greater reduction of BCR-ABL transcript with respect to pts treated with IM after IFN-α failure, giving the same complete cytogenetic response (CCR). *Aims.* We sought to determine the differences in molecular response (MR) between early and late chronic phase (CP) pts with CML who achieved a CCR after treatment with IM at the standard dose of 400mg/d. We studied 2 different cohorts of pts in CCR: a) 67/191 (35%) pts after α-Interferon (α-IFN) failure enrolled on the CML/002/STI571 protocol; and b) 53/76 (70%) pts treated front line with a combination of IM and pegilated IFN-α (PEG-IFN) enrolled on the CML/011/STI571 protocol. *Methods.* Cytogenetic response was monitored on bone marrow (BM) metaphases and MR was assessed by real time RT-PCR (TaqMan) BM and peripheral blood (PB) samples, collected at baseline, 3, 6, 9 and 12 months during the first year, and every 6 months thereafter. MR was expressed as the ratio between BCR/ABL and β2-microglobulin (β2-M) ×100. The lowest level of detectability of the method was 10^{-5}. Negative results (i.e. undetectable transcript) were confirmed by nested PCR performed 4 times (sensitivity 10^{6}). For the purpose of this analysis, a major molecular response (MMR) was defined as a BCR-ABL/β2M value <0.0001%, which turned out to be roughly equivalent to a 3-log reduction and a complete molecular response (CMR) was defined as negative (undetectable) BCR/ABL levels confirmed by nested PCR. *Results.* We observed a progressive decrease of the amount of BCR/ABL transcript in pts who achieved a CCR. At 24 months the median reduction in BCR/ABL transcript level was: 1) a 3-log reduction in late CP pts; 2) a 4-log reduction in early CP pts. In the latter group of pts MR was assessed also at 36 months. So we observed that 36 months after the first dose of IM and PEG-IFN pts who were still in CCR had the median value of BCR/ABL transcript of 0.00001% both in BM and PB. Therefore all these pts achieved a MMR. However only 8/53 (4%) pts were in CMR (undetectable BCR/ABL at least once as assessed by nested PCR). *Conclusions.* Although after 24 months of therapy front line treatment of CML pts with IM determines a major percentage of CCR in comparison of pts treated with IM after IFN failure (in our experience, 70% versus 35%, respectively) the differences in MR (reduction in BCR/ABL transcript level) observed in the 2 groups of pts were not significant. Nevertheless excellent results were obtained in both groups, with a median reduction in BCR/ABL transcript level of at least 3 log. In the pts treated with a combination of IM and PEG-INF a further reduction of BCR/ABL transcript (about another log) was observed at 36 months of treatment.

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CO-040

IMATINIB 400 MG IN LOW SOKAL RISK CML PATIENTS: RESULTS OF AN OBSERVATIONAL, MULTICENTRIC TRIAL OF THE GIMEMA CML WP

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Background. Imatinib 400 mg is the established first line treatment of chronic myeloid leukemia (CML) in chronic phase. The efficacy of imatinib in early chronic phase has been demonstrated by multicentric randomized controlled trials like the IRIS trial (O' Brien et al NEJM 348:11, 2004). Large multicentric studies aimed to evaluate the impact of imatinib 400 mg outside strictly monitored trials are not yet available. *Aims.* The GIMEMA (Gruppo Italiano Malattie Ematologiche dell'Adulto) CML Working Party opened in January, 2004, an observational study (serial n. CML/023) to investigate the efficacy of imatinib 400 mg in newly diagnosed CML patients. *Methods.* Clinical and anagraphical data were collected through a web-based system. Responses were evaluated at fixed time-points during treatment: hematologic, continuously; cytogenetic, at 6 and 12 months (local labs); molecular, at 3, 6 and 12 months. Peripheral blood samples for quantitative molecular analysis (RT-Q-PCR, Bcr-Abl/Abl × 100 - Taqman) were centralized in Bologna at 3, 6 and 12 months. *Patients.* Overall, 55 italian centers enrolled 230 (217 evaluable) low Sokal risk patients between January 1, 2004 and January, 2006. Median age was 44 yrs (range 18-69), 136 male and 81 females. 217 patients are evaluable for response at 3 months, 181 at 6 months and 118 at 12 months. The median observation time is 12 months. *Results.* At 3 months, 95% of the patients reached a stable complete hematologic response. At 6 months, 83% of the evaluable cases obtained a complete cytogenetic response (100% Ph-neg, CCgR). A major molecular response (MMR) defined as a Bcr-Abl/Abl x 100 ratio < 0.1%, was shown in 43% of CCgR patients. At 12 months, the CCgR rate was 88% and the MMR rate in CCgR patients was 59%. At 12 months, 4% of CCgR cases showed a undetectable level of transcript (ratio Bcr-Abl/Abl x 100 < 0,00001). With this short observation period, only 1 pt progressed to accelerated/blastic phase, while 2 patients were censored at the time of allogenic stem cells transplantation. *Summary and Conclusions*. The preliminary evidences of our observational trial confirm that imatinib 400 mg is a highly effective treatment for CML in early chronic phase, as far the CCgR and MMR response rates. 201 low Sokal risk patients were enrolled in the IRIS trial and received imatinib as first line treatment. The CCgR rate within 12 months was 76% with 66% of patients reaching a MMR (defined as reduction of Bcr-Abl transcript level > 3 logs; control gene Bcr) (T Hughes et al, NEJM 349:15, 2003). Our results (83% and 88% CCgR rate at 6 and 12 months, 43% and 59% MMR at 6 and 12 months) compare favourably with the IRIS trial results.

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CO-041

PH-ABNORMAL CLONES EMERGED DURING IMATINIB THERAPY: FOLLOW UP ON 31 PATIENTS FROM GIMEMA WORKING PARTY (GWP) CML REGISTRY

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Imatinib mesylate (Glivec, Novartis) is a tyrosine kinase specific inhibitor that selectively kills BCR-ABL cells *in vitro* and *in vivo*. Cytogenetic abnormalities in Ph-negative cells emerging after treatment-induced suppression of the neoplastic clone have been described. We collected clinical data on 31 such patients through the GWP in CML. To acquire insights into the origin of the Ph-negative clone as well as the clinical evolution of the coexisting Ph- and Ph+ cell populations, we have analyzed bone marrow cell segregation, cell culture and morphologic features in a subgroup of 6 patients. Patients characteristics and up to 75 months follow up are presented. The emergence of a cytogenetic abnormal clone in Ph-negative cells was evidenced in 31 patients after a median of 14.5 months after starting Imatinib. Median age was 51 years, F:M=17:14, median time from CML diagnosis 36 months. All patients have started Imatinib while in chronic phase and none of the patients had ever presented accelerated or blastic phase. Seven patients were treated with Imatinb at onset. Cytogenetics at diagnosis was characterized by the presence of Ph chromosome, except for one patient which presented with normal karyotype, but BCR-ABL B3A2 transcript. No additional abnormalities were evidenced except for one patient which presented with the Ph and a dup(1q)(q11q21). All patients achieved a good response to Glivec with 23 complete, 4 major and 4 minor cytogenetic remissions when additional abnormalities were noticed in Ph-negative cells. The clonal cytogenetic abnormalities included +8 in 14 patients, -Y in 5 patients, three del(20q), two del(5q) and del(7q), one –7, del(13q), t(6;7)(p24;q21), t(2;6)(p25;q23), with one patient presenting with both +8 and +21. The patient with dup(1q) maintained the abnormality while clearing the marrow from Ph positive cells (constitutional karyotype was normal). Retrospective analyses of stored pellet using FISH did not evidence abnormalities in previous samples. Patients that lost cytogenetic response showed that the percentage of the Ph+ cells inversely correlated to the abnormal clone. In 5 patients the abnormal clone was not evidenced in subsequent controls, suggesting the possibility that the abnormalities could be temporary. We performed cell culture on a subgroup of patients demonstrating normal growth in four patients and an abnormal growth pattern in one patient with reduced CFU formation affecting BFU-Es, CFU-GM, and colony size microclusters. FISH analyses on separated CD34+ and CD34-negative cells evidenced that the abnormal clone was present into the CD34+ compartment suggesting the stem cells involvement. FISH on cultured cells did not demonstrate a growth advantage for Ph+ cells or for the new clone. Bone marrow biopsies presented with reduced cellularity, normal differential and mild dysplastic signs as documented in patients responding to Imatinib. No increased angiogenesis was evidenced. While a longer follow up observation and laboratory analyses are required, we remark that after >4 years follow up the Ph-negative abnormal clone did not tend in our patients to evolve in MDS/AML, nor it seems to be associated with CML clonal evolution and disease progression. Hypothesis regarding the biological significance of these abnormalities are formulated.

Gene and Cell Therapy

CO-042

TRANSGENIC HLA-A*0201/CD80 K562 CELL LINE EFFICIENTLY EXPANDS EPITOPE-SPECIFIC HLA-RESTRICTED CYTOTOXIC T-LYMPHOCYTES

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Adoptive immunotherapy, which involves the transfer of antigen-specific T cells generated *ex vivo*, is a promising strategy to treat a variety of life-threatening diseases including cancer. Unfortunately, *ex vivo* generation of sufficient numbers of tumor-specific T-lymphocytes is frequently impaired by the limited availability of professional antigen presentig cells (APC). To overcome this limitation both cell- and non-cell-based artificial antigen-presenting cells have been generated.Looking for alternative APC, we have genetically modified the human chronic myelogenous leukemia cell line K562 to stably express the HLA-A*0201 molecule and the CD80 co-stimulatory molecule (K562/A*0201/CD80). We planned to evaluate whether this artificial APC can be used to expand ex vivo virus specific [Cytomegalovirus (CMV) and Epstein Barr Virus (EBV)] as well as melanoma specific (MART-1) CTLs from HLA-A2+ normal donors. K562/A*0201/CD80 cells were loaded with HLA-A2-restricted peptides derived from pp65, LMP-2, MART-1 and tyrosinase antigens. These cells were then used as APC to stimulate $CD8^+$ T-lymphocytes. Experiments were also performed in parallel using professional APCs such as DC, monocytes or CD40L activated B-blasts loaded with the same peptides. The frequency and the specificity of the T-lymphocytes stimulated with different APCs were evaluated using tetramer staining and IFN-g release assay (Elispot). We found that in a majority of cases peptide-pulsed K562/A*0201/CD80 cells were equally efficient in selecting and expanding both virus-antigen-specific and melanoma specific $CD8^+$ T-lymphocytes when compared to professional antigen presenting cells. Repeated stimulations with artificial APCs and cytokines allowed significant expansion of antigen specific CTLs. We are currently exploring whether this approach can be used to generate and expand CTL specific for weaker tumor associated antigens including PRAME and other cancer testis antigens, which are associated with several human malignancies. If successful this approach could be easily standardized according to good manufacturing practice (GMP) and used to design clinical trials in patients with several type of malignancies.

CO-043

CELLULE KILLER INDOTTE DA CITOCHINE (CIK) DOPO TRASDUZIONE CON UN RECETTORE CHIMERICO ANTI-CD19 CONTENTENTE 4-1BB(CD137), MOSTRANO ELEVATA ATTIVITA'CITOTOSSICA CONTRO LA LEUCEMIA LINFOBLASTICA ACUTA

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Background. CIK cells are a population of *ex-vivo* expanded cells with MHC-unrestricted cytotoxicity against several tumoral targets, except B-lineage Acute Lymphoblastic Leukemia (ALL). We have recently demonstrated that transduction of an anti-CD19-zeta chimeric receptor in CIK cells rendered them efficient killers of CIK-resistant ALL cells. Conceivably, the capacity to proliferate after contact with leukemic cells and to exert prolonged anti-leukemic cytotoxicity after infusion should be important to maximize the likelihood of success of this cell therapy. It was previously shown that incorporation of costimulatory molecules into chimeric receptors markedly enhances target-cell stimulated proliferation and cytotoxicity in T lymphocytes and Natural Killer cells. Aims. to identify costimulatory molecules that increase the cytolitic activity and proliferative capacity of anti-CD19-zeta receptor transduced CIK cells. Methods. CIK cells were transduced with a RD114-pseudotyped retroviral vector carrying different types of receptors: anti-CD19-zeta, anti-CD19-DAP10, anti-CD19-4-1BB-zeta and anti-CD19-CD28-zeta. A truncated form of the receptor was used as control. The cytotoxic activity of transduced CIK cells against ALL cells was detected by co-colture with the OP-1 B-lineage ALL cell line for 4 hours (short-term cytotoxic assay) or for 6 days on a mesenchimal cell layer (long-term cytotoxic assay). The recovery of ALL cells was then evaluated by flow cytometry. CIK cell proliferation was assessed in cocultures with irradiated OP-1 cells and low-dose IL-2. Results. CIK cells were efficiently transduced with the anti-CD19 retroviral vectors (average expression of GFP and chimeric receptor, 55% for all vectors tested; n = 5 each). After 4 hours of incubation, CIK cells expressing anti-CD19-zeta, anti-CD19-DAP10,anti-CD19-CD28-zeta and anti-CD19-4-1BB-zeta receptors were all strongly cytotoxic against OP-1 cells (>60% of lysis at E : T ratio 2:1 for all receptors tested). However, the benefits of adding the costimulatory molecules 4-1BB or CD28 to the receptor was evident in long-term assays with low percentages of CIK cells (E:T ratio 0.01:1). In these assays, CIK cells expressing anti-CD19-4-1BB-zeta or anti-CD19-CD28-zeta receptors had more potent cytotoxicity than cells expressing the anti-CD19-zeta receptors: in experiments with 4 donors average cell killing was 92.8% (range, 89.4%-97.6%) 93.5% (87.0%-96.8%), and 13.8% (2.9%-23.6%), respectively (p=0.001). By contrast, addition of DAP10 to the receptor did not improve cytotoxicity: average cell killing 2.3% (2.2%-2.4%). Notably, CIK cells transduced with the anti-CD19-4-1BB-zeta receptor had higher proliferative capacity in cocultures with OP1 and low dose IL-2. The average fold increase after 2 weeks of colture was 2.6 (range, 2.4-3.0) for these cells while expansion of cells transduced with either anti-CD19-zeta or anti-CD19-CD28-zeta was 1.4 (range, 1.3-1.5) and 1.7 (1.2-2.7), respectively (p=0.01). Conclusions. expression of anti-CD19-4-1BB-zeta chimeric receptors in CIK cells confers powerful and specific cytotoxic activity against ALL cells, and induces their proliferation. We suggest that anti-CD19-4-1BB-zeta expressing CIK cells may be an attractive strategy for cell therapy of ALL.

Table. Percentage of double fluorescence-positive cells at baseline (means±SD) in 3 age-groups.

Copy no.	*Baseline*	*3 mo.*	*6 mo.*	*12 mo.*	*18 mo.*	*24 mo.*	*36 mo.*	*48 mo.*	*60 mo.*
Median	0.8	1.1	2.3	7.8	3.0	8.3	6.0	2.9	43.7
Mean	19.4	5.5	9.5	10.6	8.0	48.8	49.2	8.8	45.7
Pt no.	33	32	32	28	16	17	8	6	5
Range	0-471	0-54	0-103	0-50	0-56	0-557	0-282	0-22	0-88

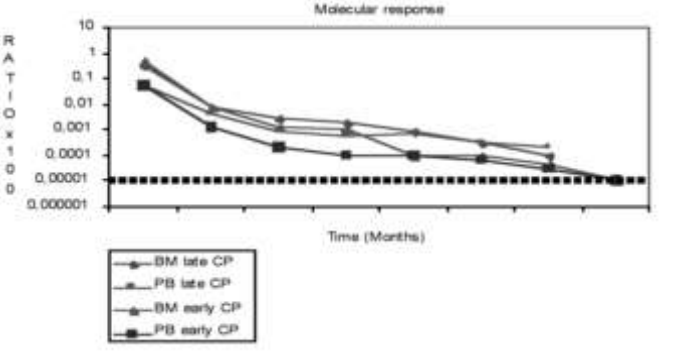

Figure.

CO-044

INHIBITION OF BURKITT'S LYMPHOMA CELLS GROWTH IN SCID MICE BY A PNA SPECIFIC FOR A REGULATORY SEQUENCE OF THE TRANSLOCATED C-MYC

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In Burkitt's lymphoma (BL) cells c-myc is often translocated in proximity to the Emiu enhancer of the Ig gene locus. This translocation causes c-myc hyperexpression and an increase in the cells proliferative capacity. We previously demonstrated that a peptide nucleic acid (PNA), linked to a nuclear localization signal (NLS), complementary to enhancer Emiu

intronic sequence (PNAEmiu), selectively and specifically blocks the expression of the c-myc oncogene under Emiu control *in vitro* in BL cells. In recent studies we confirmed the capacity of PNAEmiu to specifically inhibit c-myc expression in SCID mice when injected with BRGM cells (where c-myc is traslocated under Emiu control) but not with Ramos cells (where c-myc is translocated outside the control of the Emiu enhancer). We first showed that both pretreatment of BL cells (BRGM but not Ramos) with PNAEmiu before inoculum and chronic intravenous administration of PNAEmiu to mice already inoculated with BL cells selectively caused increased latency of tumor appearance and decreased final tumor size. The histology of tumors in the PNAEmiu treated animals demonstrated substantial areas of cell necrosis particularly around the blood vessels. We then moved to test the potential therapeutic use of a PNA in mice inoculated iv or sc with BRGM BL cells permanently transfected with the Luc gene. PNA was administrated for 2 weeks by constant infusion with osmotic micropumps implanted under the mice skin. Tumor progression was monitored as luminescence by the IVIS Xenogen imaging system. We detected significant inhibition of tumor growth by PNA with mathematical quantisation of the luminescent cells throughout the experiment. This pattern was confirmed both by autoptic examination and histological analysis when the animals were sacrified at the end of the experiment. Has to be noted that all three detection techniques revealed that systemic tumors involved the neurological system near the rachis often associated with brain infiltration and the urogenital apparatus. All our data confirm the potential therapeutic value of PNA targeted to the Emiu regulatory non coding region in BL.

CO-045

FULL PRESERVATION OF ALLOREACTIVITY IN CENTRAL MEMORY HUMAN T LYMPHOCYTES EXPRESSING A SUICIDE GENE - LINFOCITI T UMANI GENETICAMENTE MODIFICATI COL GENE SUICIDA TK MANTENGONO UN ELEVATO POTENZIALE ALLOREATTIVO

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Adoptive immunotherapy represents a powerful therapeutic approach for the treatment of cancer and infectious diseases. A compelling example is represented by donor lymphocytes infusions (DLI) after allogeneic hematopoietic stem cell transplantation (allo-HSCT). The clinical efficacy of T-cell based therapy relies not only on the ability of T cells to mediate an anti-tumor response and a protective immune response against pathogens (effector phase), but also in their capacity to persist and expand *in vivo*, providing a long-term protection from disease relapse (memory phase). Although alloreactive donor lymphocytes are very efficient in mediating malignant-cell killing (graft-versus-tumor, GvT), recognition of host antigens by donor cells often results in a life-threatening event called graft versus host disease (GvHD). To solve this double bind, we investigated the therapeutic potential of donor lymphocytes retrovirally transduced to express the suicide gene thymidine kinase of Herpes Simplex virus (TK) in patients affected by hematologic malignancies. Donor TK+ lymphocytes are sensitive to the pro-drug ganciclovir (GCV) and can thus be safely infused to patients after allo-HSCT. Preclinical and clinical studies have substantiated the concept that a timewise infusion of GCV can fully control severe GvHD and may separate the effects of GvHD from those of GvT. Encouraged by the efficacy of the suicide machinery, we investigated whether an increase in the alloreactive potential of TK+ cells is feasible, boosts the anti-tumor effect of TK cells and allows the selective control of GvHD. To this purpose, we exploited the positive effects of IL-7 in maintaining the homeostasis of memory cells. We showed that polyclonal stimulation with anti-CD3 antibodies and culture in the presence of high doses of IL-2 generates mainly CD45RA-CD62L- effector memory (EM) cells, with a mixed CD28/CD27 phenotype and a limited ability to engraft and persist *in vivo*. On the contrary, polyclonal stimulation with anti-CD3/CD28 conjugated cell sized beads and culture with low doses of IL-7 result in a high transduction efficiency and in the generation of TK+ cells with a central memory (CM) phenotype, CD45RA+/CD62L+, CD28+CD27+. These cells are able to produce high levels of IL-2 upon re-stimulation, and express persistent high levels of IL-7R α (CD127), a receptor associated to long term survival of memory T-cells. In mixed lymphocytes cultures performed with CFSE-labeled TK+ cells, CM TK+ cells showed a higher proliferative potential than EM TK+ cells. Finally, when infused in conditioned NOD/scid mice, EM TK+ cells displayed a limited ability to engraft. In contrast, CM TK+ lymphocytes showed high engraftment ability and persisted long term *in vivo*. Moreover, infused CM TK+ cells were able to preserve their characteristic phenotype several weeks after injection. Most importantly, CM TK+ cells mediated a significantly higher incidence of GvHD than EM TK+ cells. Newly developed CM TK+ cells combine a high alloreactive potential with the selective sensitivity to GCV-mediated cell death, thus providing a tool for maximal GvT and controlled GvHD.

CO-046

DECITABINE UP-REGULATES THE EXPRESSION OF THE CANCER-ASSOCIATE PRAME ANTIGEN IN EARLY CHRONIC PHASE CML CELLS

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A large body of clinical and experimental evidences indicate that the tumor cells often fail to induce specific immune response because of their inability to function as competent APC and the absence of specific antigenic tumor peptide. The cell therapy aimed at overcoming these defects by inducing or improving the antigen presenting function of tumor cells. In particular, in the contexts of CML patients, the immunotherapy might help to eradicate the residual Ph+ cells that are spared by high doses of chemoradiation of conditioning regimes of AlloSCT. In the present study, we investigate a novel CML-specific antigen, PRAME (preferentially expressed antigen of melanoma) that has been shown to be recognized by cytotoxic T cells in the context of MHC-I restriction. This tumor associated antigen is an attractive target for immune-based therapies because its expression in some hematological malignancies and its absence from normal tissues except testis. We firstly studied, by a Q-PCR assay, the expression of PRAME in the context of cell lines deriving from patients with Chronic Myeloid Leukaemia (K562, KT1), Acute Myeloid Leukaemia (HL60, Kasumi, Kg1, ME1, Thp-1) and Acute Lymphoblastic Leukaemia (BV173). Results showed a striking difference in the levels of expression of PRAME among the different cell lines, with K562 cell line expressing 1800 times the amount of PRAME mRNA expressed by KT1 cell line. The K562 and KT1 cell lines are genetically characterized by the t(9;22) translocation, that generates the P210 fusion protein. These genetic characteristic is not present, instead, in the context of the other analyzed cell lines. It demonstrates that the expression of the PRAME mRNA not depend closely on the presence of the P210 fusion protein, like, instead, showed in literature. To verify whether the expression of this gene results in the same heterogeneity even *in vivo*, we analyzed, by a Q-PCR assay, the expression of PRAME mRNA in bone marrow precursors of 30 patients with chronic phase CML; 10 of these were untreated patients at diagnosis of the disease, while the remaining had a long duration of the disease and have received previous treatments (IFN, LDAra-C, and/or hydroxyurea). None of the latter group of patients had major (complete + partial) cytogenetic conversion at the time of inclusion in the study. PRAME gene was found to be expressed, but at low levels, in 8 of the 10 newly diagnosed patients (mean level of PRAME specific mRNA was 8 fold less than the levels detected in KT1 cell line). Rather, the level of expression of the PRAME gene was found to be higher in the group of late CP-CML patients who always were positive for the expression of this antigen. In addition we found a rough correlation between the level of PRAME expression and the time from the diagnosis: the mean level of expression was 5 fold higher in the in patients with less than 60 mo of previous treatment compared with patients with more than 60 mo. Interestingly, we found that the expression level of PRAME mRNA in the group of CB-CML patients was found to be 100 fold higher than in patients at diagnosis of the disease. These results suggested that during the course of the disease, the expression of PRAME gene tends to increase. To verify whether the expression of this gene is modified by the more common used drugs in CML or by methylation status at the CpG islands of DNA, we incubated the KT1, a Ph+ cell line, and Ph+ primitive cells from untreated patients in the presence of scalar amount of IFN

(from 10 to 200 U/mL), of Imatinib (0,1 to 1,0 mM), and of hydroxyurea and of the demethylating agent decitabine (1 to 5 mM). Our findings indicated that none of the drugs with a known effect against the Ph+ cells was able to modify the expression of PRAME gene, while decitabine showed a dose-dependent effect in the inducing the expression of this gene. Indeed, we found that after 48 hours of *in vitro* incubation of Ph+ cells in the presence of decitabine, the level of PRAME specific mRNA increased up to 15 fold respect the untreated control cultures. In order to verify if the basal levels and the modulation of the expression of this gene after decitabine treatment, depend derectly on the state of gene promotor metilation, we analyzed the levels of methylation of 18 CpG dinucleotides located in the putative PRAME promoter (- 300 to +120) using sodium Bisulfite Genomic DNA Modification and DNA Sequencing. The DNA methylation analysis showed that in the patients at diagnosis of the CML, when the expression of PRAME mRNA is very low, the promoter of this gene results 40% methylated, while in patients with higher expression levels (such as in CB-CML patients) we found only 17% of the analyzed CpG in a methylated status. Moreover, the decitabine treatment of CD34+ progenitor cells from patients at diagnosis of the CML is able to decrease the mCpG levels to the 10%. Taken together, these results indicate that methylation status at specific DNA sites in involved in the expression of PRAME antigen in CML and in its gradual increase in the late CP-CML. In addition, we showed that a demethylating agent already used in the treatment of clonal hemopoietic disorders, the decitabine, is able to up-regulate PRAME gene also in early CP-CML cells, thus supporting the possible use of this drug to induce an immune-mediated control of CML.

CO-047

RECOVERY OF T CELL RESPONSES AGAINST HUMAN HERPESVIRUS-8 IS REQUIRED TO ACHIEVE CLINICAL REMISSION OF IATROGENIC KAPOSI SARCOMA AND IS ALLOWED UNDER SIROLIMUS

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Kaposi sarcoma (KS) is a tumor of hemopoietic origin, which might develop either in recipients of solid organs or in patients with rheumatic diseases, as a results of the immunosuppressive therapy. KS may occur in these immunosuppressed individuals as a result of a primary infection or a reactivation with the human herpesvirus-8 (HHV-8) infection. We have studied the T cell responses to HHV-8 latent (K12) and lytic (gB, gH, K8.1) antigens by interferon γ Elispot assay and HHV-8 viral load by real-time PCR in sequential blood samples from a well characterized series of 6 (5 HLA-A*A02 and 1 HLA-B*51) HIV negative patients with iatrogenic KS. The group included 5 post-transplant KS cases (developed in 4 renal and 1 liver recipients) and 1 case of iatrogenic KS, developed in a patient with rheumatoid arthritis (RA), treated with cyclosporine and steroids. KS regression was achieved in 5 patients (the 4 renal transplant and the RA patients) following reduction of the immunosuppression regimen based on calcineurin inhibitors (CIs), while in 1 patient (the liver transplant patient) following the switch from CIs to sirolimus. At diagnosis, T cell responses were virtually absent in all 6 patients, for both the latent and the lytic antigens. A T cell response against both the latent (60-80 spot forming cells-SFC-×10e6 cells for K12) and the lytic antigens (92-120 SFC×10^6cells for gB, 72-208 SFCs×10^6 cells for gH, and 60-180 SFCs×10^6 cells for K8.1) was observed concomitant with the remission phase of the disease obtained in the 5 patients with iatrogenic KS, following reduction of CIs and steroids. In one of these patients (the RA patient) the relapse of the tumor was associated with the progressive reduction and final disappearance of the T cell responses first against the lytic and, only later, against the latent antigens. The re-appearance of the T cell responses against the lytic antigens was associated with the persistence of iatrogenic KS and conceivably not sufficient to induce its regression in the absence of a recovered immune response against the latent antigen. In this patient, affected with a form of localized skin KS, non significant viral load variations were detected between the remission and relapse phases of the disease, which however, well correlated with the T cell responses by Elispot, which, thus, represented the only method for monitoring KS and guide therapy in this patient. In the liver transplant patient with a disseminated skin KS the switch from CIs to sirolimus, performed at KS diagnosis in December 2004, was followed by a progressive increase in circulating CD4+ and CD8+ lymphocytes. In the following months, we observed a selective increase in circulating CD4+ cells with a central memory (CD45RA-/CCR7+, CD27+/CD28+) functional (as measured by IL-2 production) phenotype. Concomitantly, a normalization of T cell repertoire was documented by spectratype analysis of the TCR Vb families, (polyclonal TCR pattern in 36% and 78% of Vb families analysed before and 6 months after switch from CI to sirolimus). T cell responses against both the lytic and the latent antigens could be detected for the first time, 4 months later (April 2005). Such T cell responses progressively increased concomitant with a progressive clinical improvement and the subsequent achievement of a complete clinical remission in July 2005. The T cell responses have been persisting in the following months (last follow-up in April 2006) associated with the maintenance of a complete clinical remission, in the absence of acute episodes of rejection or changes of liver function. This finding shows, for the first time, that sirolimus, while providing effective immunosuppression, allows the recovery of a wide and functional T cell repertoire, resulting in a protective T cell immunity against an herpesvirus and the regression of the herpesvirus associated tumor.

Molecular Oncohematology II

CO-048

DEMETHYLATION PROFILING OF CD34 POSITIVE HEMATOPOIETIC CELLS IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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Introduction. Myelodysplastic syndromes (MDS) include a heterogeneous group of clonal myeloid stem cell disorders characterized by ineffective hematopoiesis, morphologic aspects of dysplasia, peripheral blood cytopenias, and increased risk of developing Acute Myeloid Leukaemia (AML). Aberrant promoter hypermethylation of multiple genes play a pivotal role in their pathogenesis, leading to silencing of several tumor suppressor genes, as cell cycle inhibitors and inducers of apoptosis. Demethylating agents such as decitabine and azacitidine are able to revert epigenetic silencing induced by hypermethylation and are currently used to treat all subtypes of MDS. Although some of the target genes of demethylating drugs, such as p15INK4B, have been well studied and correlated to clinical response of patients, most of them remain to be identified and characterized. We aim to characterize the changes in gene expression profiling after decitabine treatment of primary CD34+ hematopoietic stem cells isolated from MDS patients and from normal bone marrow, to identify new methylation target genes in MDS in order to discover new possible markers of disease and eventually to characterize individual response to the treatment. *Methods.* CD34+ cells were isolated from the bone marrow of three patients with previously untreated high risk MDS, including: a 70 years old female with a diagnosis of Refractory Anemia with Excess of Blasts (RAEB) and deletion of 5q11-q34 and trisomy 8; a 59 years old male with a diagnosis of RAEB in transformation (RAEB-t) and a normal karyotype: a 56 years old female with a diagnosis of RAEB with a complex karyotype including deletion of 5q14-34 and trysomy 8. Health bone marrow CD34+ cells were isolated also from a patient with untreated early stage Hodgkin's disease and used as control in order to allow a comparative analysis between normal and MDS $CD34^+$ cells. $CD34^+$ cells were isolated using immunomagnetic beads and their percentage was evaluated by flow cytometry. Cells were cultured on 96-well plates in IMDM medium with L-Glutamine, antibiotics, 30% of inactivated Foetal Bovine Serum and 10 ng/mL each of IL-3, Stem Cell Factor (SCF), Thrombopoietin and FLT3-ligand. After 24 hours, decitabine was added to the culture medium to a final concentration of 1 micromolar. A corresponding amount of acetic acid was added to different wells for the mock treatment control. Each experiment was conducted in triplicate. Cells were collected after 72 hours and RNA was extracted using Qiagen RNeasy Kit and processed by two-cycle target amplification and labelling kit (Affymetrix). The biotinilated cRNA was then fragmented and hybridized on Affymetrix HG-U133A chips. Five chips were used for each patient and for the control: three for treated cells and two for mock treated cells. Microarray data were analysed by GeneSpring software version 7.2. Raw intensity values were normalized using the GC-RMA method, background-corrected and then analysed using a Volcano plot filter. In brief, genes with a fold change expression higher than two between both experimental conditions and statistically validated by a strong t-test (p<0,05; FDR correction) were selected. *Results and discussions.* Our analysis revealed 57 common genes upregulated by decitabine in all three patients. Some of the most interesting genes were: the regulator of G protein signalling 2 (RGS2), the cyclin-dependent kinase inhibitor 1A (CDKN1A, p21), the retinoblastoma-binding protein 6 (RBBP6), glycoprotein NMB (GPNMB), Epstein-Barr virus induced gene 2 (EBI 2), translation initiation factor 5B (eIF5B), two member of the Ras oncogene family (RAB7L1 and RAB31) and TRAF-associated NF-kappaB activator (TANK). Interestingly, only 2 genes were upregulated by treatment with decitabine in the control, suggesting that the demethylating drug has a minor impact on healthy $CD34^+$ cells when compared to $CD34^+$ cells of MDS patients. When comparing the expression profiling of the mock treated healthy and myelodysplastic $CD34^+$ cells, we identified 20 genes changing more than two folds. In particular, 15 of these genes were down regulated in patients with MDS suggesting the putative role of epigenetic changes in MDS. Functional significance of our data remains to be elucidated. Expression and methylation status of these genes will be investigated in a larger group of MDS patients and confirmed by real time PCR. This approach aims to characterize new genes, as methylation targets in MDS and possible markers of disease, and to identify patients responding to demethylating agents.

CO-049

AML1/ETO CAUSES HETEROCHROMATIC GENE SILENCING OF THE RETINOIC ACID SIGNALING PATHWAY IN ACUTE MYELOID LEUKEMIAS

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Silencing of key transcriptional programs by leukemia-associated fusion proteins causes myeloid differentiation-block and acute myeloid leukemia (AML). Here, we show that AML1/ETO, the commonest AML-translocation product, represses the activity of retinoic acid (RA), a transcriptional regulator of myelopoyesis. Results obtained in AML cell lines carrying an endogenous AML1/ETO (Kasumi and SKNO) or stably transfected with an HA tagged AML1/ETO (U937-AE) indicate that AML1/ETO interacts with the RA-receptor (RAR) a. Consequently, AML1/ETO localizes at RA-regulatory regions on RARb2 gene, where it recruits histone deacetylase, DNA-methyltransferase, DNA-methyl-CpG binding activities promoting repressed chromatin conformation. A link between differentiation block, heterochromatic RARb2 repression and RA-resistance is proved by the ability of either siRNA-AML1/ETO or 5-azacytidine to decrease the RARb2 promoter methylation level, increase RA-transcriptional activity and restore RA-differentiation response of AML1/ETO positive blasts. Furthermore using Southern blot analysis of genomic DNA and methylation- specific PCR (MSP) we found that the RARb2 promoter region containing the β-RARE and transcription start site is methylated in samples from 7/9 AML-M2, 9/10 AML-M4 (6/19 are AML1/ETO positive), and a region located in the 5'portion of RARb2 exon 1 is methylated in 9/9 AML-M2, 8/10 AML-M4 (8/19 are AML1/ETO positive). Neither of these RARb2 regions was found methylated in CD34+ normal hematopoietic precursors. Accordingly, RARb expression was detectable in normal CD34+ cells but not in any of the 19 AML cases analysed. In summary these results indicate the transcriptional silencing of RA-signaling pathway as a common mechanism of leukemogenesis, which is also caused by non-APL AML-associated fusion protein. Thus, the restoration of the epigenetic function of RARs might represent a powerful target for the transcriptional/differentiation therapy of AML.

CO-050

KNOCK-DOWN OF COLD SHOCK DOMAIN PROTEIN A (CSDA) INCREASES β GLOBIN GENE EXPRESSION IN K562 CELL LINE.

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In order to identify and study regulatory factors putatively involved in γ-globin gene expression, we examined the reticulocyte transcriptome of three siblings, all of them resulted to be homozygous for the β + IVSI-6 (C'T) mutation, even though presenting different levels of HbF and varied severity of β-thalassemia intermedia conditions. Extensive sequence analysis previously performed on putative regulatory regions within the β-globin gene cluster[1] had revealed the same genetic background in all the siblings and excluded HPFH mutations. To explore the hypothesis that genetic determinants unlinked to the β-globin gene cluster were responsible of the different γ-globin gene expression levels, we used the differential display approach to examine the reticulocyte mRNAs differently expressed in the three siblings and found different expression levels for the cold shock domain protein A (CSDA), reported to act as transacting factor for several hematopoietic genes[2] and particularly to interact with the -200 promoter region of the G-γ-globin gene, where some HPFH mutations fall.[3] Quantitative real time PCR

analysis of CSDA and γ-globin gene mRNA levels was performed on reticulocyte RNAs to confirm data obtained by differential display and revealed an inverted correlation between HbF values and CSDA mRNA levels, comparable to that found between CSDA and γ-globin gene mRNAs. To analyze the role played by this factor, we examined the effects produced on γ-globin genes expression by CSDA transient RNA interference in K562 cell line. Relative amounts of CSDA and γ-globin mRNAs were determined by quantitative Real time PCR in cells treated with specific or unspecific siRNAs. Results showed an average of two-fold increased level of γ-globin mRNA when CSDA expression was silenced at about 40-50%. Our data indicate that a quantitative defect of CSDA expression may produce a significant persistence of HbF in adult life and provide further insights into the involvement of CSDA in the control of γγ-globin genes expression, thus suggesting possible novel targets for gene therapy in hemoglobinopathies.

References

1. Zhi-Hong Lu et al., Blood 1996; 87: 1604-11
2. P. Coles et al. Nucleic Acids Res 1996; 12: 2311-17
3. Horwitz M. et al. JBC 1994; 269: 14130-39

CO-051

ABERRANT METHYLATION OF SOCS-1 (SUPPRESSOR OF CYTOKINE SIGNALLING 1) AND SHP-1 (SH2-CONTAINING PHOSPHATASES 1) GENES IN IMMUNODEFICIENCY-RELATED LYMPHOMAS

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DNA hypermethylation of CpG sites in the promoter regions of tumor suppressor genes is a frequently acquired epigenetic event in the pathogenesis of many human cancers, resulting in transcriptional repression of these genes. Suppressor of cytokine signalling-1 (SOCS-1) and SH2-containing phosphatase 1 (SHP-1) proteins are members of a family of negative regulators of cytokine signalling and inhibit the pathway mediated by Janus kinase (JAK)/signal transducers and activators of translocation (STAT). The SOCS-1 gene is located on chromosome 16p12-p13.1. The single coding exon (exon 2) transcribes a 1215 bp mRNA, which in turn encodes 211 aminoacids. A CpG island is located within the promoter region upstream of the untraslated exon 1. The SHP-1 gene is located on chromosome 12p13, consists of 17 exons and it is expressed primarily in hematopoietic cells from the hematopoietic cell-specific P2 promoter. SHP-1 encodes a small cytoplasmatic protein phosphatase (38 kD) that acts as a negative regulator in terminally differentiated hematopoietic and immune cells. Here we tested the involvement of SHP-1 and SOCS-1 inactivation in immunodeficiency-related lymphoma. Thirty tumor samples of AIDS-related non-Hodgkin lymphoma (AIDS-NHL) and 20 post-transplant lymphoproliferative disorders (PTLD) were analyzed for SOCS-1 and SHP-1 aberrant methylation by methylation-specific polymerase chain reaction (MSP). The tumor panel included 10 AIDS-Burkitt lymphoma (AIDS-BL), 12 AIDS-diffuse large B cell lymphoma (AIDS-DLBCL), 8 AIDS-primary effusion lymphoma (AIDS-PEL), 3 PTLD-BL, 3 polymorphic PTLD (P-PTLD), 5 PTLD-centroblastic (PTLD-CB) and 9 PTLD-immunoblastic (PTLD-IB). DNA from the peripheral blood of 3 healthy blood donors and 10 healthy bone marrow donors was used as negative control, and DNA from several cell lines (HeLa, MCF7) was used as positive control. For SOCS-1, we analized the CpG island located within exon 2 that has been demonstrated to correlate with exon 2 methylation and loss of gene expression. For SHP-1 gene, we used MSP primers selected for the hematopoietic-specific P2 promoter. SOCS-1 hypermethylation occurred in 3/30 (10%) AIDS-NHL, in particular 1/12 (8%) AIDS-DLBCL and 2/8 (25%) AIDS-PEL. No SOCS-1 hypermethylation was detected in AIDS-BL (0/10). SHP-1 aberrant methylation occurred in 17/30 (57%) AIDS lymphoma: 4/10 (40%) AIDS-BL, 6/12 (50%) AIDS-DLBCL and 7/8 (87%) AIDS-PEL. With respect to PTLD, the aberrant methylation rate was 6/20 (30%) for SOCS-1 and 15/20 (75%) for SHIP-1 gene. In particular, SOCS-1 and SHPI-1 hypermethylation was more frequently detect in PTLD-IB than in other PTLD categories. EBV-infection was present in 7/11 (64%) PTLD. Among EBV-positive patients, SHP-1 was hypermethylated in 7/11 (64%) cases and unmethylated in the remaining 4/11 (36%) cases. Overall, these data indicate that inactivation of SHP-1 and SOCS-1 genes by aberrant hypermethylation may have a role in the pathogenesis of lymphoma in HIV-infected patients and in PTLD.

CO-052

CEL WITH T(1;5)(Q21;Q33) GENERATING A NEW TPM3/PDGFRB FUSION TRANSCRIPT

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Background. Receptor tyrosine kinases such as ABL1, PDGFRB, PDGFRA, C-KIT, are promiscuous genes i.e. they rearrange with several, diverse partners. Under normal conditions, their catalytic domain activation is mediated by dimerization or oligomerization. Fusion to oligomerization-promoting moieties derived from their gene partners triggers tyrosine kinase activity in chronic myeloproliferative disorders like chronic eosinophilic leukaemia (CEL) which is typically associated with FIP1L1-PDGFRA/del(4)(q12). Rare cases of CEL show 5q31-q33 rearrangements and/or PDGFRB involvement. *Aim.* To identify the molecular event underlying a t(1;5)(q21;q33) translocation of a CEL case. *Materials and Methods.* In 1992, a 21-year old man with CEL showed a 46,XY,t(1;5)(q21;q33) karyotype in 28/29 metaphases. He obtained a major cytogenetic response with α-interferon treatment, which was administered for ten years. In April 2002 imatinib was started and the patient achieved hematological and FISH remission (last check-up, January 2005). To study the 5q33 region, metaphase FISH was performed with cosmid 9-4 for the 3' PDGFRB and cosmid 4-1 for the 5’ PDGFRB. The long arm of chromosome 1 was examined with a panel of 17 DNA clones mapping at bands 1q21-q23.The 5q33 breakpoint disrupted PDGFRB and the 1q21 breakpoint fell within clone RP11-205M9 which contains the following genes: TPM3, C1F43 and UBAP2L. Patient RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) from a bone marrow sample taken at diagnosis and retro-transcribed using Thermoscript RT-PCR System (Invitrogen). Nested RT-PCR studies sought the fusion trancript TMP3/PDGFRB in the first round of amplification with primers TPM3_425F (AGGTGGCTCGTAAGTTGGTG) and PBGFRB_2369R (TAGATGGGTCCTCCTTTGGTG) and in the second with primers TPM3_425F and PDGFRBR1 (TAAGCATCTTGACGGCCACT). RT-PCR also sought the reciprocal PDGFRB/TMP3 chimeric transcript using primers PDGFRB_1686F (CCGAACATCATCTGGTCTGC) and TPM3v2_1158R (GGATTCGATTGCTGCTTCAG) followed by nested PCR with primers PDGFRB-1810F (AGGAGCAGGAG TTTGAGGTG) and TPM3_919R (GGTGGTGAAAGGAGAAAGCA). PCR products were cloned in pGEM-T easy Vector System (Promega, Madison, WI) and sequenced. *Results*. RT-PCR studies revealed a TPM3/PDGFRB chimeric transcript joining exon 7 of TPM3 with exon 11 of PDGFRB and the reciprocal chimeric transcript joining exon 10 of PDGFRB with exon 8 of TPM3. *Conclusions.* We identified TPM3, as a new PDGFRB/5q33 translocation partner in CEL. TPM3 is an actin-binding protein whose muscle isoform mediates myosin-actin response to calcium ions in skeletal muscles and whose non-muscle isoform is found in cytoskeletal microfilaments. Known TPM3 rearrangements in human malignancies are: anaplastic cell lymphomas and inflammatory myofibroblastic tumours with t(1;2)(q25;p23) producing the TPM3-ALK fusion and papillary thyroid and colon carcinomas where TPM3 rearranges with the nearby NTRK1 gene. Moreover, an heterozygous TPM3 germline mutation has been associated with the autosomal dominant form of nemaline myopathy (OMIM 609284). In our patient, the constitutively activated TPM3/PDGFRB tyrosine kinase is imatinib mesylate-sensitive.

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CO-053

IMMUNOPHENOTYPIC CHARACTERISTICS AND *IN VITRO* CLONAL EXPANSION CAPACITY OF CIRCULATING AND BONE MARROW-DERIVED ENDOTHELIAL CELLS FROM HEMATOLOGICAL DISORDERS: A COMPARISON WITH ENDOTHELIAL-LIKE CELLS

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So far, several studies have described the immunophenotypic profile of circulating endothelial cells (CEC), and circulating endothelial progenitors cells (EPC) in various pathologic and physiologic conditions, while a few investigations dealt with cultured bone marrow (BM)-derived and peripheral blood(PB)-derived EPC, that have the capacity of forming *in vitro* endothelial colonies (CFU-En). In this study, the reactivity of a wide panel of monoclonal antibodies (MAbs) directed against EC- associated markers was assessed by 4 colors flow cytometry protocol and immunocytochemical analysis in fresh and cultured PB and BM-derived samples obtained from healthy subjects (NS) and hematological malignancies (HM, myelodysplastic syndrome and multiple myeloma). Moreover, the *in vitro* clonogenic expansion capacity of circulating and BM-derived EPC, CEC and the so called e*ndothelial like cells* (ELC) was comparatively evaluated in NS and HM. Our results showed an increased number of CEC (showing a CD31+/CD61-/CD34+/ CD45-/ CD146+/CD105+/ CD29+ phenotype) in fresh PB and BM samples in HM, as compared with NS. The frequency of BM-derived EPC was very low in NS and exhibited a wide distribution pattern among HM. Our results further showed that cultured PB-CEC and BM-EPC could be distinguished on the basis of their *in vitro* clonogenic potential, that resulted higher for PB-CEC, than that of BM-EPC. Interestingly, EC-like cells (ELCs) were frequently detected in fresh PB and BM samples, but they lacked an *in vitro* expansion capacity. The multicolour flow cytometry analysis showed that *in vitro* expanded 7-AAD-CD45- PB-EPC/CEC, and BM-EPC could also be distinguished on the basis of their phenotype since they differ for the expression of CD34+, KDR, AC133 and CD117 antigens. The immunphenotypic profile of the so called ELC was quite characteristic, being CD45+/CD34- and showing co-expression of several monocytic and endothelial markers. In conclusion, the combined use of a 4-color cytofluorimetry protocol, and cell culture expansion capacity assessment, could be regarded as a valid tool, capable to identify different endothelial subpopulations in both normal and HM samples.

Acute Leukemias

CO-054

MODULATION OF TRYPTOPHAN CATABOLISM BY HUMAN AML CELLS RESULTS IN THE CONVERSION OF CD4+CD25- INTO CD4+CD25+ T REGULATORY CELLS

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Indoleamine 2,3-dioxygenase (IDO) enzyme, which catalyzes the conversion of tryptophan into kynurenine, has been identified as a novel immunosuppressive agent by inhibiting T-cell proliferation. IDO expression has been recently demonstrated in tumors of non-hematopoietic origin and in some subsets of normal immunoregulatory cells after induction by interferon (IFN)-γ. Here we show that IDO protein is neither constitutively expressed in normal CD34+ hematopoietic stem/progenitor cells nor is induced by IFN-γ. Conversely, 13 out of 48 (27%) newly diagnosed acute myeloid leukemia (AML) patients constitutively express IDO resulting in tryptophan depletion and kynurenine metabolite production. Functionally, IDO-expressing AML cells inhibit allogeneic T-cell proliferation by tryptophan degradation and the addition of IDO-specific inhibitor 1-methyl-tryptophan (1-MT) completely restores T-cell alloreactivity. *In vitro*, IDO+ AML cells increase the percentage and the absolute number of CD4+CD25bright T cells expressing surface CTLA-4 and Foxp3 mRNA and this effect is completely abrogated by 1-MT. Purified CD4+CD25+ T cells obtained from co-culture with IDO+ AML cells are anergic and inhibit naive T-cell proliferation, thus fulfilling the functional and phenotypical characteristics of T suppressor/regulatory cells (Tregs). Co-culture with IDO+ AML cells results in the conversion of CD4+CD25- into CD4+CD25+ T cells and the addition of 1-MT completely abrogates this effect. In mice, intrasplenic injection of IDO+ leukemia/lymphoma A20 cells induces the conversion of CD4+CD25- into CD4+CD25+ Tregs, which is blocked by 1-MT. These data indicate that IDO expression is a novel marker associated with leukemic transformation and identify IDO-mediated catabolism as a T-cell inhibitory effector mechanism in human AML cells.

CO-055

ERK1/2 PHOSPHORYLATION IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS: PROGNOSTIC ROLE AND *IN VITRO* EFFECTS OF MEK INHIBITION

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Extracellular signal-regulated kinase-1/2 (ERK1/2) is frequently found constitutively activated (p-ERK1/2) in hematopoietic diseases, suggesting a role in leukemogenesis. Recently, small molecules targeting this pathway have been investigated to evaluate their use as novel therapeutic strategies. The aim of this study was to assess the expression and clinical role of p-ERK1/2 in adult acute lymphoblastic leukemia (ALL). In 131 primary samples from adult *de novo* ALL patients enrolled in the GIMEMA LAL2000 protocol and evaluated by Western blot analysis and flow cytometry (D-value), constitutive ERK1/2 activation was found

in 34.5% of cases, resulting significantly associated with higher WBC values (r=0.17; p=0.05). In a multivariate analysis, p-ERK1/2 expression was an independent predictor of CR (p=0.027). We evaluated the *in vitro* effects of the MEK inhibitor PD98059 on 23 fresh ALL samples: among samples characterized by constitutive ERK1/2 activation, a significant down-regulation was observed in 8/12 (p=0.012) reaching in 4 a complete signal abrogation. However, neither cell cycle changes, nor induction of apoptosis, occurred. In conclusion, p-ERK1/2 is expressed in a proportion of adult ALL and is associated with poor prognosis. A more effective approach toward MEK inhibition must be explored to evaluate whether it may represent a new therapeutic target in ALL.

CO-056

BREAST CANCER RESISTANCE PROTEIN (BCRP) EXPRESSION AFFECT EVENT-FREE SURVIVAL IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS

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The transporter breast cancer resistance protein (BCRP) is a member of the ATP-binding cassette which has been recently described as a protein involved in the multidrug resistance (MDR) phenotype. There are currently no reports concerning the role of this protein in adult acute lymphoblastic leukemia (ALL). The aim of this study was to evaluate the frequency of BCRP expression, its correlation with other MDR-related proteins and their prognostic role in 118 untreated adult ALL patients uniformly treated according to the GIMEMA protocol LAL 2000. BCRP protein expression was detected by flow cytometry using the monoclonal antibody (MoAb) BXP-34 (Kamiya, Seattle, WA); results were analyzed according to the Kolmogorov-Smirnov (KS) statistic test (D-value). Detection of BCRP in the cell lines MCF7 pcDNA3 and MDA231 pcDNA3 showed a D-value of 0.10±0.07 and 0.10±0.07, respectively. In contrast, the cell lines MCF7 pcDNA3 clone 8 and MDA231 pcDNA3 clone 23 that overexpress BCRP were characterized by a D-value of 0.44±0.21 and 0.32±0.11, respectively. Analysis of primary ALL samples showed a BCRP expression (D-valueβ0.20) in 86/118 (72.9%) cases, with a mean value of 0.36±0.22 (range 0.00-0.87) in the overall population. BCRP expression resulted associated with age (p=0.027). No significant difference was found with other clinical characteristics. The multidrug resistance associated protein (MRP1), analyzed by the MoAb MRPm6, resulted expressed in 70.1% of samples (MFI1.20); the MDR1/P-glycoprotein-170 (MDR1), evaluated with the MoAb MRK16, was present in 28.2% of cases (D-value0.05). Samples analysed for both BCRP and MRP1 expression (115/118) showed a significant correlation (R=0.54; p=0.0001): 20.9% of cases were negative for both proteins, while 46.1% expressed both BCRP and MRP1. In addition, MRP1 negative cases showed lower BCRP levels (mean 0.30±0.15, range 0-0.60) compared to MRP1 positive (mean 0.38±0.11, range 0-0.87) (p=0.017). BCRP expression did not correlate with MDR1 expression. None of these proteins separately influenced the achievement of complete remission (CR). In contrast, BCRP/MRP1 co-expression influenced response to induction treatment: 94.4% (17/18) double negative (BCRP-/MRP1-) for both proteins achieved CR, while 69.6% (48/69) of cases positive for one or two proteins (BCRP-/MRP1+ or BCRP+/MRP1- or BCRP+/MRP1+) responded to induction treatment (p=0.034). To further investigate the prognostic significance of BCRP, a Kaplan-Meier analysis was performed for event-free survival (EFS): interestingly, among 99 evaluable patients a significant difference (p=0.03) was observed with a median EFS of 8.4 months (95% CI, 5.63-11.28) in BCRP+ cases (72 cases) compared to BCRP- patients (27 cases) for whom median EFS has not been achieved. Multivariate analysis confirmed the unfavorable prognostic role of BCRP positive cases on EFS (p=0.072; OR 1.87, 95% CI, 0.947- 3.707). In summary, our study shows that adult ALL frequently express BCRP. In patients treated with the GIMEMA LAL 2000 protocol EFS was unfavorably affected by BCRP expression, while co-expression of MRP1 and BCRP was associated with failure to induction treatment. The expression of these proteins may contribute to explain the overall poor outcome of adult ALL patients, suggesting that therapeutic strategies based on overcoming drug resistance remain an important goal to be pursued.

CO-057

PREVALENCE AND PROGNOSTIC SIGNIFICANCE OF FLT3 MUTATIONS IN ACUTE MYELOID LEUKEMIA: ASSOCIATION OF ITDS WITH POOR OUTCOME IN PATIENTS WITH NORMAL CYTOGENETICS

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Acute Myeloid Leukemia (AML) is a difficult disease to treat, and better treatments are needed. Molecular targeted therapy represents a novel therapeutic approach. Activating mutations of FMS-like tyrosine kinase 3 (FLT3) are present in approximately one third of patients with de novo AML and have been implicated in its pathogenesis. The leukemic blasts of most AML patients have the internal tandem duplications (ITDs) in the juxtamembrane region or point mutations in Asp835 and Iso836 codons in the activation loop of the kinase domain (TKD) of the FLT3 receptor. Both mutations result in constitutive FLT3 receptor activity and may play a significant role in leukemogenesis. In this study we have analyzed the incidence and type of FLT3 mutations in a large series of newly diagnosed AML patients. Furthermore, we have evaluated the prognostic impact of FLT3 mutations. The FLT3/ITD was determined by polymerase chain reaction (PCR). The mutations of D835 and I836 codons were determined by PCR followed by restriction enzyme digestion (PCR-RFLP). For the estimation of the statistic significance of the differences in the clinical-biological characteristics, between the mutated patients and wild-type patients, it has been used the Student's test t for independent data. The probabilities of overall survival (OS) and disease free survival (DFS) were analysed by Kaplan-Meier method; the differences of OS and DFS, between the mutated patients and wild-type patients, were assessed using the log-rank test. Both FLT3/ITD and FLT3/TKD mutations were found in 15%. Dual mutations were found in 2% of 126 patients. Among the FAB subtypes of AML, the rate of FLT3 aberrations was higher in M4 (27%) and M5 (26%). FLT3/ITD was associated to leukocytosis (106.8×10^9/L vs 30×10^9/L in FLT3-wt, p=0.015) and high percentage of circulating blast cells (82% vs 42% in FLT3-wt, p<0.0001). Differently, FLT3/TKD mutations were not associated with high white blood cells count and blast cells percentage. FLT3 mutations were more prevalent in patients with normal karyotype (51%). In this group, DFS and OS were significantly inferior for patients with FLT3/ITD than patients withouth mutations (0 vs 5, p=0.0032; 5 vs 9, p=0.049, respectively). We have identified the FLT3/ITD as an independent poor prognostic factor in AML patients with normal cytogenetics. Therefore, targeting FLT3 mutations represents a potential therapeutic target for AML. These results suggest that new treatment modalities, such as therapy with a FLT3 tyrosine kinase inhibitor, are clearly needed for this group of patients with *standard risk* profile.

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CO-058

SPONTANEOUS APOPTOSIS AND MULTIDRUG RESISTANCE IDENTIFY TWO DIFFERENT BIOLOGIC SUBSETS FOR OUTCOME PREDICTION IN ACUTE MYELOID LEUKEMIA (AML)

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P-glycoprotein (PGP)-mediated multidrug resistance (MDR) upregulation and anti-apoptotic proteins (bcl-2, bcl-xl, Mcl-1) overexpression represent two key mechanisms explaining the high rate of treatment failure in AML. Gene expression profiling studies have identified as discriminator genes for a poor prognosis genes involved either in drug resistance or in apoptosis block (Wilson CS et al, Blood, 2006). Moreover, the today availability of third-generation PGP inhibitors and bcl-2 antisense oligonucleotides moved us to consider the impact of a multidrug resistance phenotype and/or apoptosis on AML prognosis. Therefore, from 1995 to 2005, a large series of 379 patients, affected by *de novo* AML, except FAB M3, median age 58 years, treated with intensive chemother-

apy regimens (EORTC AML10, AML12 and AML13), were tested. The principal aims of our study were: 1) to correlate bax/bcl-2 ratio, as a measure of spontaneous apoptosis, with PGP expression as a measure of multidrug resistance phenotype; 2) to demonstrate that bax/bcl-2 ratio and PGP are biologically and clinically independent events. PGP, bcl-2 and bax proteins were determined by multicolor flow cytometry. PGP was evaluated with the monoclonal antibody MRK-16 as percentage and the threshold was set at >20%. Bax/bcl-2 ratio was obtained by dividing mean fluorescence intensity (MFI) of bax/MFI bcl-2. The threshold was set at the median value >0.3 (range 0.01-9.1). Two hundred-nineteen patients were bax/bcl-2 ratio positive (219/379; 57.8%) and 241/379 (63.6%) were PGP positive. There was a close correlation between higher CD71 expression and MDR positive staining by flow cytometry (175/241; $p<0.00001$), showing that overexpression of the transferrin receptor (CD71), a sign of increased blast proliferation in AML, is strictly linked to a MDR phenotype. Moreover, a significant correlation was found between a higher bax/bcl-2 ratio and a higher MDR (150/241; $p=0.023$), demonstrating that a MDR phenotype is linked to a higher amount of spontaneous apoptosis. A significant lower complete remission (CR) rate was found in patients with lower bax/bcl-2 ratio (41.6% vs 69.8%, $p<0.00001$) or higher PGP (48.2% vs 70.5%, $p=0.0001$). Overall survival (OS) was significantly shorter either in pts with lower bax/bcl-2 ratio (0% vs 16% at 3.5 years; $p<0.00001$) or higher PGP (2% vs 15% at 4 years; $p=0.0001$). A longer disease free survival (DFS) was observed either in pts with higher bax/bcl-2 ratio (17% vs 0% at 2.7 years; $p=0.0001$) or lower PGP (13% vs 0% at 3.9 years; $p=0.001$). Bax/bcl-2 ratio and PGP showed additive prognostic properties, since higher bax/bcl-2 ratio plus lower PGP identified an AML subset at better prognosis with regard to CR (84.5% vs 33.3%; $p<0.00001$), OS (41% vs 0% at 2.4 years; $p<0.00001$) and DFS (46% vs 0% at 1.2 years; $p=0.00001$). Interestingly, patients with only a higher PGP presented an intermediate outcome, while patients with only low apoptosis showed a worse outcome. In order to establish the superior and independent prognostic value of bax/bcl-2 ratio from PGP, we investigated MDR + (241 pts) and MDR - (138 pts) AML subgroups. As a matter of fact, a lower CR rate was found in patients with lower bax/bcl-2 ratio either within MDR+ (31.4% vs 61.1%, $p=0.00005$) or within MDR- subsets (55.5% vs 86.4%, $p=0.0003$). Lower bax/bcl-2 ratio was associated both with a shorter OS and DFS in MDR+ (0% vs 8% at 2.4 years, $p=0.001$; 0% vs 15% at 1.2 years, $p=0.003$) and, more significantly, in MDR- (0% vs 34% at 3.5 years, $p<0.00001$; 0% vs 32% at 2.7 years, $p=0.0008$) patients. On the other hand, PGP was not able to distinguish AML subsets at different prognosis within the lower bax/bcl-2 ratio subgroup. Interestingly, within the higher bax/bcl-2 ratio subset, PGP+ pts showed a significant lower CR (61% vs 86%, $p=0.0005$), OS (5% vs 34% at 4 years, $p<0.00001$) and DFS (0% vs 23% at 3.9 years, $p=0.0008$). The independent prognostic value of bax/bcl-2 ratio was confirmed in multivariate analysis with regard to CR ($p=0.00002$), OS (hazard ratio: 21.3, $p=0.000004$) and DFS (hazard ratio: 23.9, $p=0.000001$). In conclusion, the lack of an important correlation between bax/bcl-2 ratio and MDR confirms the independence of apoptosis and multidrug resistance pathways. Furthermore, the capacity of bax/bcl-2 ratio of clearly identifying patients at different prognosis within the MDR+ and MDR- subgroups implies that apoptosis has an intrinsic more relevant clinical significance than a multidrug resistance phenotype. Infact, PGP didn't show any prognostic significance in pts with higher levels of antiapoptotic proteins, even if the favorable prognostic impact of higher bax/bcl-2 ratio was greatly reduced by PGP positivity. That has to be taken in account focusing future research and therapeutic strategies mainly on apoptosis-inducer drugs in order to improve outcome in AML.

CO-059

INTEGRATIVE MICROARRAY-BASED STUDY OF GENE EXPRESSION AND GENOTYPE DATA IN CHILDHOOD ALL WITHOUT KNOWN GENETIC ABERRATIONS

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Childhood acute lymphoblastic leukemia (ALL) is characterized by high biological and genetic heterogeneity. Beside the risk stratification based on the presence of the most prognostic relevant translocations, approximately 20-25% of childhood ALL patients cannot be classified according to cytogenetic detectable hallmarks. Recent studies applied single nucleotide polymorphism (SNP)analyses shoving that Loss of Heterozygosity (LOH), with or without loss of genetic material, is frequently related to childhood ALL and AML. Moreover, there is an increasing interest in evaluating the results of gene expression profiling to identify new genetic subgroups. Indeed, the efficient exploitation of gene expression databases requires not only computational tools for management, analysis, and functional annotation of primary data, but also integrating lists of modulated genes with of other sources of genomic information, such as gene sequence, locus or structural characteristics. In particular, integration between expression profiles and chromosomal localizations could be effective in detecting gene structural abnormalities such as genomic gains and losses and/or translocations, and their consequences. We applied an integrated approach composed by immunophenotype definition, DNA index estimation, PCR (and/or RT-PCR) and cytogenetics, to exclude patients with known molecular and cytogenetics aberrations. Aim of the study was to identify criptic abnormalities in childhood ALL patients by performing an integrative analysis of gene expression and gene copy number variation data. The patients included in our study met the following inclusion criteria: a) B-cell precursor childhood ALL b) DNA index 1; c) negativity at t(4;11), t(12;21), t(9;22), t(1;19) RT-PCR screening; d) cytogenetics revealing normal karyotype or not technically feasible due to lack of metaphases or poor chromosome morphology. Moreover, all patients were analyzed for gene expression in the context of the International, multilaboratory MILE (Microarray Innovation in Leukemia) project, and they were classified as a single class by the *MILE class predictor*, which includes cases not classified in other known subgroups. As clearly emerges from these inclusion criteria, these patients represent a subgroup without any known genetic classification. As a *proof-of-principle*, we analyzed the first series of 23 patients. After the inclusion screening, patients genomic DNA has been analyzed by the Affymetrix GeneChip Mapping 100K SNP array platform in order to identify regions of LOH and gene copy number variation with a nearly genome-wide coverage. In parallel, total RNA was extracted and purified, and biotin-labeled cRNA was synthesized and hybridized on HG-U133Plus2 Probe Arrays in accordance with the Affymetrix protocols. The presence of del(9)(p21) was found in 7 out of 23patients, with an homozygous commonly deleted region involving CDKN2A and CDKN2B genes in 4/23. Additional hemyzygous losses of the short arm of the chromosome 9 were found in two patients. Two patients showed the hemyzygous deletion of chromosome 12p13.2, involving the ETV6 gene. Other microdeletions were found in single cases, involving potentially interesting genes. Four patients were normal with respect to the presence of copy number changes. Three patients presented LOH without copy number change, indicative of segmental Uniparental Disomy (UPD) in different chromosomal regions. Gene expression data of samples positive and negative to the presence of del(9) or del(12p) was also analyzed in the context of the physical localization of genes in the genome to verify if the deletion in the chromosome reflects in chromosomal regions with transcriptional imbalances. Chromosomal regions with modulation of the gene expression signals have been identified using a non-parametric model-free statistical method, named locally adaptive statistical procedure (LAP). The method, which accounts for variation in the distance between genes and gene density, is based on the computation of a standard statistic (e.g. SAM t-statistic) as a measure of the difference between deleted and non-deleted samples. For each chromosome, the statistic is locally smoothed using a non-parametric estimation of regression function over the positional coordinate. Differentially expressed regions are identified using a permutation procedure (e.g., B=100000 permutations). In particular, gene positions are randomly shuffled and the randomly generated statistics smoothed to generate the null smoothed distribution. This empirical null distribution is finally used to estimate the q-value measure of significance. The integration of genomic variation and gene expression profiling might may be useful to identify new hidden genetic lesions, and to learn how critical regulators of tumor are linked to genomic alterations in cancer cells.

CO-060

IDENTIFICATION OF A NOVEL MUTATION IN THE EXON 11 OF NUCLEOPHOSMIN (NPM1) GENE

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Background. Mutations occurring in nucleophosmin (NPM1) gene result in an abnormal cytoplasmatic expression of NPM1. To date, all genetic modifications described are clustered in the exon 12 and represent the most frequent acquired abnormalities in de novo AML with normal karyotype. All mutated NPM1 alleles detected so far carry at their C-terminal a short stretch of hydrofobic amino acids, named nuclear export signal (NES) motif. The loss of tryptophan residue 290 and the gain of the NES-motif are considered crucial for the cytoplasmic accumulation of NPM mutant protein. We recently identified a novel mutation in the exon 11 of NPM1 gene (submitted). Aims: Because of the strong association between nucleotidic sequence of the C-terminal portion and the functionality of the NPM protein, we performed a DHPLC-based analysis and subsequent sequencing of the exons from 9 to 12 of the gene in 98 AML patients, excluding M3 subtype. *Methods.* Bone marrow or peripheral blood samples were collected at diagnosis. Reverse transcription-PCR (RT-PCR) was done for the analysis of NPM1. The C-terminal portion of the gene was spliced in two amplificaton fragments. Exon 12 was amplified using the couple of NPM1_1112R (5'-cctggacaacatttatcaaacacggta-3') and newly designed NPM1_870F (5'-tggttctcttcccaaagtggaa-3') primers. DHPLC runs were performed at 55.3° C, as suggested by the Navigator(e)TM software predicted melting curve (Ver 1.6.0). A second amplication step of exons 9, 10 and 11 of the NPM1 gene was carried out for homoduplex samples at the first DHPLC round. Direct PCR was conducted by using the NPM1_1112R and newly designed NPM1_658F (5'-gaaaaagcgccagtgaagaaa-3') primers. DHPLC analysis was performed at 54° C and 55° C. The amplicons showing an heteroduplex profile were sequenced directly in both strands. An allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) was performed to confirm the identity of the newly identified mutation in exon 11 (Figure 1). *Results.* Twenty-six AML tested patients (26.5%) were mutated. We identified 2 novel sequence variants, here named VI1 and VI2 (Table 1). Variant VI1 (958_961insTGTT) leads to the acquisition of the most frequent NES motif type (LxxxVxxVxL). The mutation type VI2 consists of the insertion of 8 nucleotides at position 902, in the middle part of exon 11 (902_909insGGCGCCTA). This new insertion leads to the creation of a stop codon at the level of the amino acid number 275 (Met274Stop). So, the predicted truncated protein consists of 274 amino acidic residues instead of 294 of the wild type. *Conclusions.* To date, variant VI2 is the only mutation described mapping outside the NPM1 exon 12. The predicted aberrant protein lacks the NPM C-terminal NES motif and do not contain neither tryptophan 288 nor 290. Further investigations would be important to understand the consequence of the lacking of the C-terminal NES motif in the nucleolar-cytoplasmic shuttle properties of the mutant protein VI2.

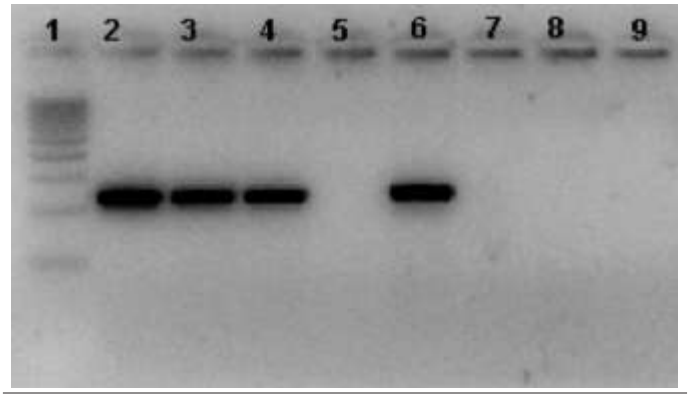

Figure 1. Detection of mutation VI 2 by ASO-PCR. Lane 1: DNA ladder 100 bp; Lane 2: Patient's cDNA amplified with normal primer; Lanes 3, 4: Control cDNA amplified with normal primer (NP); Lane 5: Mix 1 reaction control; Lane 6: Patient's cDNA amplified with mutation specific primer (ASO); Lanes 7, 8: Control cDNA amplified with mutation specific primer (ASO); Lane 9: Mix 2 reaction control.

Table 1. Novel NPM1 mutations found in this study. The wild-types NPM sequences are aligned with mutant variants VI1 and VI2. GenBank Accession n. NM_002520.

Mutant type	*Exon*	*Sequence*
WT	12	952-gatctc----tggcagtggaggaagtctctttaa
VI1	12	952-gatctc**TGTT**tggcagtggaggaagtctctttaagaaaa
WT	11	892-gccaaattca--------tcaattatgtgaagaat
VI2	11	892-gccaaattca**GGCGCCTA**tcaattatgtga

Chronic Lymphoproliferative Disorders

CO-061

ANALYSIS OF IMMUNOGLOBULIN VARIABLE GENES IN HIV-RELATED NON HODGKIN LYMPHOMA REVEALS IMPLICATIONS FOR DISEASE HISTOGENESIS AND PATHOGENESIS

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Non-Hodgkin lymphomas (NHL) represent a frequent complication of HIV infection and a major source of morbidity and mortality among patients affected by AIDS. Although the incidence of NHL in AIDS patients has diminished since the introduction of highly active antiretroviral therapy, NHL constitute an increasing proportion of AIDS-defining events diagnosed in recent years. HIV-related NHL (HIV-NHL) are a suitable model to study lymphomagenesis in the context of disrupted immunosurveillance. In particular, molecular analysis of the immunoglobulin variable region (IGV) genes can provide insights into the nature of the cell of origin and its clonal history following neoplastic transformation. We investigated a panel of 110 HIV-NHL, including 61 systemic HIV-NHL, 30 HIV-related primary central nervous system lymphomas (HIV-PCNSL), 6 HIV-related primary effusion lymphomas and 13 HIV-related plasmablastic lymphomas (PBL) of the oral cavity. Cases were studied for usage, mutation frequency and intratumoral heterogeneity of clonal IGV heavy (IGHV) and IGV kappa (IGKV) and lambda (IGLV) chain gene rearrangements. Moreover, to assess the role of antigen in HIV-NHL pathogenesis, we analyzed the mutational profile and CDR3 structure of IGV genes utilized by HIV-NHL. Results were compared to a database of 330 IGV rearrangements from aggressive NHL of the immunocompetent host (IC-NHL) as well as to the normal B-cell repertoire. A functional IGHV rearrangement was identified in 107/110 (97%) HIV-NHL; a functional IGKV rearrangement was found in 26/65 (40%) cases and a functional IGLV rearrangement in 39/65 (60%) cases. Somatic hypermutation in IGV genes was observed in 101/110 (92%) HIV-NHL. The average mutation frequency was 8.9% for IGHV genes and 5.8% for IGV light chain genes. Unmutated IGV rearrangements preferentially associated with HIV-PBL ($p<0.05$) and HIV-PCNSL with immunoblastic features. Among IGV mutated cases, average mutation frequencies did not differ among HIV-NHL pathologic categories. Analysis of IGV gene usage showed a significant overrepresentation of the IGHV4 family (18/37; 49%) and a significant under representation of the IGHV3 family (11/37, 30%) in HIV-diffuse large B-cell lymphoma (HIV-DLBCL) compared to aggressive IC-NHL ($p<0.001$) and to normal B-cells ($p<0.002$). In particular, IGVH4-34 was the IGHV gene most frequently rearranged (18/110; 16%) and was overrepresented in HIV-NHL compared to normal B-cells (5%; $p<0.05$). The IGKV4-1 gene was the IGKV segment most frequently rearranged (7/26; 27%) in HIV-NHL and its usage was biased compared to normal B-cells (5%; $p<0.05$). The single IGLV gene most frequently encountered was IGLV6-57 (9/39; 23%). Analysis of the distribution of replacement and silent mutations in IGHV sequences showed a tendency to conserve framework (FR) sequences and maintain antigen binding in 69/101 (68%) cases. A higher than expected number of CDR replacement mutations, suggesting selection for high affinity antigen binding, occurred in 31/101 (31%) cases. Analysis of intraclonal heterogeneity showed the presence of ongoing mutations in only 2 out of 15 HIV-NHL (1 HIV-Burkitt-like lymphoma and 1 HIV-DLBCL). In 12/15 cases, intraclonal heterogeneity was absent. Implications of these data are multifold. First, most HIV-NHL categories are highly mutated in IGHV and/or IGV light chain genes, documenting derivation from B-cells persistently subjected to the GC reaction and suggesting a potential role for antigen stimulation in the pathogenesis of these lymphomas. This hypothesis is supported by the finding of antigen binding preservation in the majority of HIV-NHL. The absence of IGV gene mutations in a fraction of HIV-PBL and HIV-PCNSL suggests a different histogenetic and pathogenetic pathway for these lymphomas. Second, because IGHV4-34 and IGKV4-1 have been linked to autoimmune clones, the preferential usage of these genes in a fraction of HIV-NHL may suggest a role for antigen stimulation of preneoplastic B-cells with polyreactive and/or autoreactive activity. Finally, at variance with IC-NHL, the presence of intraclonal heterogeneity is a rare finding in HIV-NHL, suggesting a derivation from B-cells that have concluded the GC-reaction.

CO-062

TELOMERE LENGTH IDENTIFIES TWO DIFFERENT PROGNOSTIC GROUPS AMONG VH-UNMUTATED B-CLL PATIENTS

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Introduction. Telomere restriction fragments (TRF) length is an effective indicator of histopatogenesis and a well-known prognostic indicator in B-Cell Chronic Lymphocytic Leukemia (B-CLL). Long telomeres have been associated with VH-mutated status, while VH-unmutated patients are regarded as having uniformly short telomeres. However, little is known on cases in which discordance exists between these two parameters. *Patients and methods*. 201 B-CLL patients were analyzed for TRF length and VH mutational status. All samples were taken before starting an anti-CLL treatment. M/F ratio was 124/77. Median age was 62 years (range 33-93). Median follow-up since diagnosis was 30 months (range 12-281). According to Binet staging, 121 patients were A, 39 B and 41 C. CD38 and ZAP-70 expression was performed by flow cytometry on 157 and 111 patients, respectively. FISH analysis for Ch11, 12, 13, 17 was performed on 161 patients. TRF length was evaluated by Southern Blot assay as previously described (Ladetto M *et al.,* Blood 2004). VH mutational status was evaluated by direct sequencing according to standard methods. Survival curves were calculated with the Kaplan-Mayer method. In particular we calculated Overall Survival (OS), starting from diagnosis to death or the last follow-up and Progression Free Survival (PFS), defined as the time ranging from the end of the first treatment to the start of a second-line treatment or the last follow-up. *Results.* TRF length analysis was performed in all samples with a median of 6014 bp (1465-16762). There was no correlation between TRF length and patient age, sex or stage. VH sequencing was successful in 183 patients (91%): 118 were VH-mutated and 65 were VH-unmutated. As expected, a correlation was found among these two biological parameters ($r^2=0.1994$, $p<0.0001$). VH-unmutated patients had shorter telomeres (median 4120 bp) compared to that of VH-mutated patients (median 7152 bp) ($p<0.0001$). A mathematical model has been employed to analyze the distribution of TRF length in VH-mutated and unmutated patients. While mutated patients had a homogeneous distribution, unmutated patients showed a bimodal distribution, with no patients falling between 4137 and 4710 bp. This allowed to discriminate two different groups among unmutated patients: 38 (58%) with short telomeres (concordant pts) and 27 (42%) with long telomeres (discordant pts). Concordant and discordant patients had similar patterns of VH usage and similar levels of homology (H) to the germline IgH sequence (i.e. H =100% vs H <100% and >99% vs H <99% and >98%). In addition, they showed no difference for all the available biological and clinical parameters. However, the two populations had significant differences in terms of clinical outcome. Among VH-unmutated patients, those who had long TRF length showed a significantly better clinical outcome than those who had short telomeres. In fact median OS was of 214 and 81 months respectively ($p<0.05$, Figure A), and median PFS was of 29 and 13 months respectively ($p<0.05$, Figure B). Moreover, VH-unmutated patients with long telomeres had a clinical behavior similar to that of VH-mutated patients (median OS: 281 months and median PFS: 54 months, p=n.s.) The role of telomere length as survival predictor was further supported by performing univariate and multivariate analysis indicating that TRF length is the most powerful prognostic indicator among those considered (age, sex, Binet, CD38, ZAP70, FISH and mutational status). *Conclusions.* Our data demonstrated that: 1) TRF length is heterogeneous among B-CLL patients and correlates with VH-mutational status. However, discordance between VH-mutational status and TRF length was observed in 42% of VH-unmutated patients; 2) when discordance exists, TRF length allows to discriminate two different prognostic groups among VH-unmutated patients; 3) multivariate analysis strongly confirms the prognostic impact of TRF length.

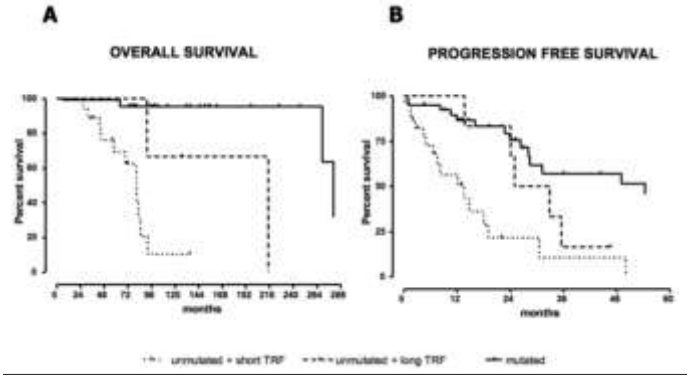

Figure.

CO-063

LONG TERM MONITORING OF CANCER-FREE SUBJECTS CARRYING NON LYMPHOMA ASSOCIATED BCL-2/IGH REARRANGEMENTS (NLABR): PROLONGED PERSISTENCE OF CLONAL POPULATIONS POTENTIALLY RELATED TO FL

Mantoan B, De Marco F, Drandi D, Pollio B, Astolfi M, Vallet S, Ricca I, Dell'Aquila M, Francese R, Aguzzi C, Pagliano G, Monitillo L, Santo L, Critelli R, Rocci A, Lobetti Bodoni C, Cristiano C, Borchiellini A Schinco PC, Boccadoro M, Tarella C, Ladetto M[1]

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Introduction. Non-lymphoma associated Bcl-2/IgH rearrangements (NLABRs) are frequently observed in cancer free-subjects. We recently observed that NLABR-positive clones can persist up to 60 days (Ladetto et al, J Clin Oncol 2003). However the long-term kinetics and potential pre-neoplastic role of NLABR-carrying cells are unknown. To define the natural history of NLABR-positive clones, long term monitoring of cancer-free subjects carrying these lesions has been performed. *Methods.* 125 subjects undergoing periodical blood examinations for warfarin therapy were screened for the bcl-2/IgH translocation. NLABR-positive clones were monitored using both nested and real time-PCR according to previously published approaches (Ladetto et al Exp Hematol 2001). Sequence homology of NLABRs has always been confirmed by direct sequencing of nested PCR products. *Results.* 16 NLABR-positive subjects were identified out of 125 (12.8%) subjects, and were monitored at least every six months for a median time of 22 months (range 6-50). In eight subjects (50%), the NLABR-positive clone disappeared promptly, all follow-up samples being PCR negative with the exception of two specimens in which a second transient but unrelated rearrangement was amplified. Overall, persistent NLABRs were monitored for a median time of 18 months (range 6-47). In five subjects persistent NLABRs were detected in all follow-up specimens. In three subjects persistent NLABRs were detected only in a proportion of samples, while the others were PCR-negative. Both transient and persistent rearrangements were indistinguishable from Bcl-2/IgH rearrangements usually observed in FL in terms of breakpoints, presence of N- insertions and preferential JH6 usage. All samples scoring PCR postive by nested PCR, were also quantified by real- time PCR showing a range from $5,4\times10^4$ cr $/10^6$ to dg $2,8\times10^0$ cr$/10^6$ dg. The number of NLABR-positive cells appeared to be rather stable in subjects with persistent NLABR-positive clones. Subjects having mixed PCR-positive and PCR-negative results had a smaller tumor burden if compared to those constantly PCR-positive ($p<0.001$). Studies on selected populations showed that NLABR-positive cells were CD19-positive. This demonstrates that NLABRs are exclusively associated to B-cells. Discussion: This suggests the existence of a follicular lymphoma (FL)-related clonal expansion of undetermined significance, which might be either a pre-malignant or a non-malignant counterpart of FL. Since NLABRs occurs in more than 10% of healthy subjects, this condition is expected to be highly prevalent in the general population (as observed in MGUS and CLUS) and of potential relevance for the pathogenesis of FL.

CO-064

ABERRANT SOMATIC HYPERMUTATION IS INVOLVED IN TRANSFORMATION OF FOLLICULAR LYMPHOMA AND CHRONIC LYMPHOCYTIC LEUKEMIA TO DIFFUSE LARGE B-CELL LYMPHOMA

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The molecular mechanisms involved in histologic transformation of follicular lymphoma (FL) and B-chronic lymphocytic leukemia (B-CLL) to diffuse large B-cell lymphoma (DLBCL) are heterogeneous and largely unknown. Here we explored whether aberrant somatic hypermutation (SHM), leading to the acquisition of new mutations in PIM-1, PAX-5, RhoH/TTF and c-MYC genes, is involved in transformation from FL or B-CLL to DLBCL. This study was based on: i) 18 paired specimens from 9 grade 1-2 FL patients collected at FL diagnosis and at the time of transformation to DLBCL; and ii) 18 paired specimens from 9 B-CLL patients collected at B-CLL diagnosis and at the time of transformation to DLBCL. All cases were classified according to the WHO classification. All B-CLL specimens were from PBMC. All FL and DLBCL specimens were from lymph node biopsies. Mutational analysis of the PIM-1, PAX-5, RhoH/TTF and c-MYC genes was performed on selected regions that contain > 90% of mutations found in DLBCL. Immunoglobulin heavy chain variable region (IGHV) rearrangements were amplified with family-specific primers. Amplicons were directly sequenced. IGHV sequences were considered mutated if deviation from the corresponding germline gene was greater than 2%. At the time of transformation from FL, 5/9 (55.5%) DLBCL acquired a total of 25 new mutations in one or more proto-oncogenes. In three cases, a single mutation was already present in the FL phase and was preserved after transformation. Transformation was characterized by acquisition of novel mutations of PAX-5 in 3/9 cases, RhoH/TTF in 3/9 cases, PIM-1 in 2/9 cases and c-MYC in 1/9 cases. Two cases acquired two novel mutations in PIM-1 and c-MYC coding exons, leading to aminoacid substitutions with potential functional consequences. During transformation from B-CLL, 2/9 (22.2%) DLBCL acquired 3 new mutations in one or more proto-oncogenes. None of the cases displayed evidence of aberrant SHM during the B-CLL phase. Transformation was characterized by acquisition of novel mutations of PAX-5 in 1/9 cases, RhoH/TTF in 1/9 cases and PIM-1 in 1/9 case. A functional VDJ rearrangement was obtained in 8/9 FL/DLBCL pairs. In all pairs, the FL and the DLBCL phases displayed identical VDJ rearrangements, indicating a common clonal origin. Mutation analysis of IGHV genes revealed that all FL and DLBCL pairs were somatically mutated. In 6/8 FL/DLBCL pairs, some IGHV mutations were found in the FL phase but not in the DLBCL phase, while some mutations appeared only in the DLBCL phase. At variance with SHM in IGHV genes, all proto-oncogene mutations due to aberrant SHM in the FL phase were maintained at the time of DLBCL transformation, concomitantly with the appearance of additional mutations. This finding may suggest that aberrant SHM is active only in the subclone that subsequently gives rise to DLBCL, where it may confer selective growth advantage. A functional VDJ rearrangement was obtained in all 9 B-CLL/DLBCL pairs. In all pairs, the B-CLL and DLBCL phases displayed identical VDJ rearrangements, indicating a common clonal origin of the tumor samples. Mutation analysis revealed germline IGHV genes in 7/9 pairs. The remaining two pairs displayed mutations at a frequency of 3.3% and 4.5%, respectively. No DLBCL transformed from B-CLL acquired new IGHV mutations. In one DLBCL transformed from FL and in 2 DLBCL transformed from B-CLL, novel proto-oncogene mutations consistent with aberrant SHM were acquired despite the absence of novel mutations in IGHV genes. This finding supports the hypothesis that aberrant SHM is due to a qualitative, rather than to a quantitative defect of this process. Our findings demonstrate that acquisition of novel mutations due to aberrant SHM associates with DLBCL transformation. Aberrant SHM may activate PAX-5, RhoH/TTF, PIM-1, and c-MYC by at least two modalities. First, because mutations cluster around the 5' untranslated region of the gene, it is conceivable that these mutations may deregulate gene transcription by affecting specific regulatory regions. Second, in cases of DLBCL transformed from FL, a

subset of newly acquired mutations introduced aminoacid substitutions in c-MYC and PIM-1, thus potentially altering the biochemical and/or structural properties of the proteins.

CO-065

NOVEL MUNC13-4 MUTATIONS IN CHILDREN AND YOUNG ADULT PATIENTS WITH HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

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Familial hemophagocytic lymphohistiocytosis (FHL) is a genetically heterogeneous disorder characterized by constitutive defects in cellular cytotoxicity resulting in fever, hepatosplenomegaly, cytopenia, and the outcome is fatal unless treated by chemo-immunotherapy followed by hematopoietic stem cell transplant. Since 1999 mutations in the perforin gene giving rise to this disease have been identified, however these only account for 40% of cases (FHL2). Lack of a genetic marker hampers the diagnosis, suitability for transplantation, selection of familial donors, identification of carriers, genetic counselling and prenatal diagnosis. Mutations in the Munc13-4 gene have recently been described in patients with FHL, defined as FHL3. Thirty patients with HLH diagnosed according to current diagnostic criteria, in which PRF1 mutations had been ruled out, underwent a genetic study. Genomic and mRNA sequences of Munc13-4 gene were retrieved from the National Center for Biotechnology Information (NCBI; LOC201294 of the genome annotation program, genomic contig NT_010641, model mRNA XM_113950). Genomic DNA was prepared from the peripheral blood samples obtained from the patients and their family members. To analyze the Munc13-4 gene, 32 exons and adjacent intronic regions were amplified obtaining 21 fragments directly sequenced, in both directions, with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). In 15 of the 30 families studied, 12 novel and 4 known Munc13-4 mutations were found, spread throughout the entire gene. Among novel mutations, C2650T introduced a stop codon; 441del A, 532del C, 3082del C, and 3226ins caused a frameshift, and seven were missense mutations. Median age of diagnosis was 4 months, but 6 patients developed the disease after 5 years of age and one as a young adult of 18 years. Central nervous system involvement was present in 9/15 patients, natural killer activity was markedly reduced or absent in 13/13 tested patients. Chemo-immunotherapy was effective in all patients. Munc13-4 mutations are found in one half of non-FHL2 patients. Most mutations fall within the functional domains of the protein. The clinical features of FHL3 patients are not peculiar, although CNS involvement appears an important component of the disease. Since these patients may develop the disease during adolescence or even later on, not only pediatric but also as adults, hematologists should include FHL-2 and 3 in the differential diagnosis of young adults with fever, cytopenia, splenomegaly and hypercytokinemia.

CO-066

MOLECULAR CHARACTERIZATION OF DUP(1)(Q) IN ACUTE LYMPHOID LEUKEMIA AND IN BURKITT LYMPHOMA

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Background. Chromosome 1 long arm duplications, dup(1)(q), are recurrent changes in chronic myeloproliferative disorders, in L3 subtype acute lymphoid leukemia (ALL), and in Burkitt's lymphoma (BL). They may occur as a primary abnormality, i.e. isolated chromosomal change, or as a secondary abnormality in leukemic subclones at diagnosis or during disease progression. Boundaries of 1q duplications and their molecular significance are not definitely established. *Aim.* Molecular characterization of 1q chromosome undergoing duplication in ALL and BL. *Materials and methods.* We studied three ALL and one BL at diagnosis. Cytogenetics showed a dup(1)(q) in the main leukemic clone of two cases of ALL. In the third case of ALL and in the BL with t(8;14)(q24;q32), the dup(1)(q) was identified by FISH. Metaphase FISH was performed with probe D1Z5 (Oncor) for the α-satellite region and puc 1.77 for the heterochromatic sequences of chromosome 1. A panel of 38 DNA clones mapping at 1q21-1q25 were applied. Analysis of 5-10 cells bearing the dup(1)(q) was carried out using a fluorescence microscope (Provis, Olympus) equipped with a CCD camera (Sensys, Photometrics) run by Smartcapture software (Vysis, Olympus). *Results.* A common duplicated 1q21.2 region extending 93Kb and corresponding to clone RP11-212K13 was identified despite variations in the centromeric and telomeric dup(1)(q) breakpoints and duplicated segment extension. *Conclusions.* This study defines the critical 1q duplicated region in ALL and BL. In this region three putative oncogenes/tumor suppressor genes have been mapped: the SF3B4 gene, an RNA-binding protein; the MTMR11 gene, a member of the lipid phosphatase; and the ZA20D1 gene which encodes for a cytoplasmic deubiquitinating enzyme. On-going molecular studies are assessing whether and how, if any, of these genes are linked with dup(1)(q21.2). *Acknowledgments. Fondazione Cassa di Risparmio di Perugia; Associazione Sergio Luciani, Fabriano; PRIN-MIUR (Ministero per l'Istruzione, Università e Ricerca Scientifica) Italy.*

CO-067

IDENTIFICATION OF DUPLICATION AT 17Q12 AS AN EARLY EVENT IN CUTANEOUS T-CELL LYMPHOMAS.

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Background. Mycosis Fungoides (MF) and its leukemic variant, Sézary syndrome (SS) are common primary cutaneous T-cell lymphomas (CTCL), an heterogeneous group of extranodal non-Hodgkin lymphomas. Conventional cytogenetics showed complex karyotypes with undefined markers, but specific changes have not been identified yet. Molecular cytogenetic studies of these rare T-cell proliferations are scarce. Recently a FISH study identified multiple copies of Her2/neu gene as a recurrent event in SS (Utikal et al., Leukemia Research, 2006;30:755-760). Comparative Genomic Hybridization (CGH) is a powerful approach to overcome limits of conventional cytogenetics, which may only investigate proliferating cells in metaphase. We selected 7 cases of CTCL, three MF and four SS, and used Comparative Genomic Hybridization (CGH) to detect clonal chromosomal imbalances. *Materials and Methods.* CGH experiments were performed using DNA extracted from paraffin embedded cutaneous biopsies. Briefly, 50 slices were washed two times in xilene to remove paraffin. Tissues were dehydrated in 100% ethanol, air dried, and resuspended in lysis buffer (50 mM Tris-HCl pH 8.5/1mM EDTA/0.5% Tween 20). After overnight incubation at 37°C, 0.6 mg proteinase K, and 10 microliters of SDS 10% solution per milliliter of buffer were added to each sample, and kept at 37°C for 24 hours. DNA extraction was performed according to standard phenol-chloroform method. Reference DNA was extracted from a healthy donor. Test and reference DNA were green- or red-labelled with a nick-translation method. After hybridization and washes, CGH slides were analysed with PathVysion digital image analysis system (Vysis). *Results.* 4/4 cases of Sézary Syndrome, and 1/3 cases of Mycosis Fungoides showed genetic imbalances. Gains (11) were more frequent than losses (2). Complete or partial duplication of the long arm of chromosome 17 was found in all cases. This duplication was the sole genomic event in three out of the five cases (4 SS and 1 MF). The other imbalances were gains at chromosome 1q (1 case), 2q (1 case), 7p and 7q (1 case), and 12p. Losses involved chromosome 10 as deletion of the entire long arm (q) in one case, and of band q23 in a second case. Among three Mycosis Fungoides cases only one case had an abnormal profile, with gain of 1q21-q41 and of 17q at q11.2-q22. Interestingly, a minimal duplicated region at 17q12, corresponding to the mapping of Her2/neu gene, was common to all cases showing genomic imbalances (4 SS and 1 MF). From this study, genomic duplication at 17q12 emerges as a typical primary lesion in the pathogenesis of Sézary syndrome. Moreover the presence of the same duplication in Mycosis Fungoides strongly suggests that 17q12 duplication is an early clonal event in CTCL.

POSTERS

Stem Cells and Hematopoietic Growth Factors

PO-001

INVOLVEMENT OF THE UROKINASE-MEDIATED PLASMINOGEN ACTIVATION SYSTEM IN HEMOPOIETIC STEM CELL MOBILIZATION

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The urokinase plasminogen activator (uPA) is a serine protease that activates plasminogen to plasmin and binds to a specific high affinity cell surface receptor, uPAR. uPAR is anchored to the cell membrane through a glycosyl-phosphatidylinositol tail. uPAR can be shed by the cell-surface, and soluble uPAR (suPAR) has been detected in plasma and urine from patients affected by various diseases. Both cell surface and soluble uPAR can be proteolitically cleaved generating truncated forms of uPAR (c-uPAR) and suPAR (c-suPAR). Cleaved suPAR or a uPAR-derived peptide containing the SRSRY sequence (uPAR84-95), are able to activate receptors for fMet-Leu-Phe (fMLP) inducing migration of monocytes and basophils and hematopoietic stem cells (HSC). We have recently reported that G-CSF administration to healthy donors up-regulates uPAR expression in circulating myeloid precursors and monocytic cells, and increases s-uPAR and c-suPAR levels in sera. c-suPAR and its derived peptide (uPAR84-95) induced *in vitro* migration of marrow (BM)-HSCs by activating the high affinity fMLP-receptor (FPR). The uPAR peptide also inactivated CXCR4, the chemokine receptor primarily responsible for HSC retention in BM. These *in vitro* findings prompted us to examine the role of c-suPAR in leukemic cell migration and to investigate whether c-suPAR may directly contribute to HSC mobilization *in vivo*. We demonstrated that uPAR84-95 may stimulate M1 cell migration and may inactivate murine CXCR4; thus, it potentially could influence HSC retention in bone marrow, allowing their mobilization into the circulation. In mouse, intraperitoneal administration of uPAR84-95 induced rapid leukocytosis, which was associated with increased circulating $CD34^+$ cells. *In vitro* colony assays confirmed that uPAR84-95 mobilized hematopoietic progenitors, showing an absolute increase in circulating colony forming cells. uPAR84-95 mobilizing activity was comparable to that of G-CSF; however, neither synergistic nor additive effect was observed combining the two molecules. Our findings show, for the first time, that chemotactic suPAR fragments, detected in human biological fluids, are able to regulate cell adhesion and migration *in vitro* and *in vivo*, thus suggesting that their increase in some diseases, such as acute leukemias, is not a side effect of increased uPAR expression, but likely contributes to tumor cell migration. Moreover, the finding that uPAR84-95 exerts a HSC mobilizing activity similar to that of G-CSF, suggests a potential utility of the cleaved form of suPAR, or its derived chemotactic peptide, in the strategies to optimize HSC mobilization, perhaps in G-CSF poor mobilizers.

PO-002

NEOPLASTIC CIRCULATING ENDOTHELIAL CELLS IN HEMATOLOGIC MALIGNANCIES

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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 microvascular ECs in B-cell lymphomas 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. *Aims.* To clarify if, in different hematologic malignancies with known cytogenetic aberrations, CECs are tumor-derived. *Methods.* We studied 21 patients with different hematologic malignancies (6 MM, 2 CML, 5 AML, 1 ALL and 7 CLL). To isolated CECs, we used a dual step immunomagnetic sorting by means of CD45 and CD146 antibodies. By using immunomagnetic sorting in combination with CD45, we first eliminated all hematopoietic cells, which are CD45 positive, without affecting the EC component, which is characteristically CD45 negative. We then sorted CECs by means of CD146, an antigen expressed almost exclusively on ECs and absent on hematopoietic cells. To confirm the EC commitment, we then performed additional phenotypic studies with antibodies recognizing endothelial and neoplastic cells. FISH analysis was finally performed on sorted CECs with different commercially available probes in dual colour experiments. *Results.* In all experiments more than 95% of immunomagnetically sorted cells were of EC origin as demonstrated by phenotypic analyses. After immunomagnetic selection less than 0.5% of cells were CD45+ while CD14 was expressed in 0.1% of all immunomagnetically sorted CECs. More than 95% of immunomagnetically sorted CECs expressed VEGFR2, vWf, CD144 and UEA-1 lectin. Very few immunomagnetically sorted CECs expressed antigens expressed on neoplastic cells (CD138, CD38, CD33, CD19, CD5). FISH analysis showed that a significant proportion of CECs was tumor-derived because they harbored the same genetic lesion as observed in neoplastic cells. The fraction of CECs showing the cytogenetic aberration averaged 20% (range, 11-34%, 200 cells observed in each case). The majority (>85% of CECs presented features of EPCs because they expressed CD133, a marker gradually lost during EC differentiation and absent in mature ECs. Overall, 98.0% of CECs with genetic lesions were CD133 positive. *Conclusions.* These findings suggest that in many hematologic malignancies CECs are in part tumor related and with EPC features. These CECs may contribute to tumor neovasculogenesis and possibly to the spreading and progression of the disease. It is possible to speculate that neoplastic CECs may have arisen from a common hemangioblast precursor that can give rise to both neoplastic cells and ECs or alternatively through a process of dedifferentiation of a already committed cell into a cell with EPC characteristics followed by a redifferentiation into a terminally differentiated EC. Disguised neoplastic cells may then mimic functional CECs and contribute to tumor neovasculogenesis.

PO-003

DIOXIN *IN VITRO* EXPOSURE OF THE HEMATOPOIETIC CELL LINE K562 DEMONSTRATED TRANSCRIPTION MODULATION OF GENES INVOLVED IN MAPK PATHWAY, CALCIUM SIGNALLING AND APOPTOSIS

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2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) arises from the chlorination of phenolic substrates or the partial combustion of organic materials in the presence of chlorine sources, and interacts with the aryl hydrocarbon receptor (AhR), a member of the erb-A family that also includes the receptors for steroide, thyroid hormones, peroxisome proliferators and retinoids. When bound to dioxin, AhR can bind to DNA and alter the expression of some genes, such as cytokines and growth factors. TCDD is highly toxic, and causes a wide range of biochemical and pathological changes in mammalian and non-mammalian species, including carcinogenesis, hepatotoxicity, teratogenesis, cardiovascular disease, diabetes, chloracne, cancer, and immunotoxicity. Epidemiological data based on the population exposed to TCDD after the 1976 industrial accident in Seveso, Italy has shown an increased risk of hematological cancer and could support a possible role of TCDD in the neoplastic transformation of hemopoietic stem cells. Approximately 20 years after the accident, a population-based study of randomly selected subjects from the exposed zones revealed an excess of lymphohemopoietic neo-

plasms: the risk of Hodgkin's disease was high in the first 10-year observation period, whereas the greatest increase in non-Hodgkin lymphoma and myeloid leukemia occurred after 15 years. As no data regarding the transcriptional effect of TCDD exposure on hemopoietic cells are available, we analysed the effect of escalating TCDD doses on a human hemopoietic cell line, K562, in order to elucidate the biological effects and the gene expression modulation induced by dioxin, by means of cell culture (CFU-GM colony growth assay) and macroarray analyses. The analysis identified 59 genes whose expression was significantly different between treated and untreated cells. Analysis of the functional classes of genes affected by TCDD exposure showed that modulated genes can be grouped in 8 functional groups. The groups with more regulated genes resulted the ionic channel/membrane integral proteins group (12 elements), the ATP binding/protein kinases group (11 elements), and the transcriptional factor group (9 elements). The other groups contained genes involved in oncogenesis (7 elements), apoptosis (6 elements), DNA repair (4 elements), cell cycle (2 elements), cell adhesion (2 elements). Six genes remained unclustered. The genes identified are variously involved in MAPKs pathways, in regulation of intracellular calcium, apoptosis, and cytoskeleton. These are the first data available on transcriptional regulation mediated by TCDD in an hemopoietic precursor cell line. Most importantly, the contemporary and cohordinated involvement of all these pathways has never been described whatsoever. Therefore our result suggest that in an hemopoietic precursor cell system, TCDD exposure may perturbate cellular membrane equilibrium, possibly affecting the fluxes of various molecules and in particular of calcium, alter the apoptotic equilibrium by affecting different genes involved in this processes, and may alter intracellular signaling mediated by MAPK cascade, affecting the relative abundance of different transcripts.

PO-004

PKC-EPSILON EXPRESSION AND TRAIL-INDUCED APOPTOSIS OF CD34-DERIVED ERYTHROID CELLS FROM REFRACTORY ANEMIA PATIENTS

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Introduction. Myelodisplastic syndromes (MDS) are a group of clonal disorders of haematopoietic stem cells characterized by a variable risk of developing a secondary acute myeloid leukaemia (AML). The increased apoptotic index in MDS is associated with an increased proliferative fraction leading to a peripheral blood cytopenia with an hypercellular bone marrow (ineffective haematopoiesis). However, in late MDS and AML arising from MDS, the apoptotic index appears to decrease when compared to early MDS; during the clinical progression of the disease, in fact, apoptotic signals decrease while anti-apoptotic signals increase.The members of TNF super family seem to play a role in the pathogenesis of MDS: in particular it has been demonstrated that Fas and TRAIL might by involved in the early death of erythroid precursor in the bone marrow. Protein kinases C are involved in a number of basic cell functions such as proliferation, survival and differentiation. In particular, PKC-epsilon has antiapoptotic and prosurvival effects, promoting haematopoietic cell proliferation in the absence of IL-3, and is modulated under erythropoietin (EPO)-driven CD34 cell differentiation. *Material and Methods.* Cell isolation and culture: $CD34^+$ cells were isolated from bone marrow aspirates of healthy donors (HD) and patients with refractory anemia (RA), and differentiated in *X-vivo* medium supplemented with IL-3, SCF and EPO. The level of erythroid differentiation was evaluated quantifying the surface expression of glycophorin-A and CD71 by flow cytometry. Analysis of PKC-epsilon expression: the expression and the activation of PKC-epsilon was analyzed by Western blot with anti-PKC-epsilon and anti-phospho-PKC-epsilon (serine 792) rabbit sera, respectively. TRAIL-induced apoptosis: at day 11 of EPO-differentiation, cells were treated with TRAIL (250 ng/mL for 48 hours) ans their viability was evaluated by flow cytometry analysis of Annexin V and propidium iodide staining. Modulation of PKC-epsilon expression: to modulate PKC-epsilon levels in CD34 cells derived from RA patients, we transfected the cell cultures with pCMV-PKC-epsilon vector (expressing PKC-epsilon under CMV-derived promoter) or with pCMV-mutated PKC-epsilon (expressing a mutated PKC-epsilon form, with a single point mutation deleting kinase activity). *Results.* EPO increased cell sensitivity to TRAIL-induced apoptosis in the initial phases of erythroid differentiation of $CD34^+$ cells derived from HD. At day 9 of differentiation, EPO induced PKC-epsilon accumulation that is known to increase the expression of Bcl-2, thus protecting cells at this stage of differentiation from TRAIL-induced apoptosis. CD34 cells derived from RA patients, on the contrary, appear far more sensitive to TRAIL-induced apoptosis than those from HD, while no resistance to TRAIL appeared with cell differentiation. A differential modulation of PKC-epsilon in the CD34 cells derived from RA patients is discussed, in the more general frame of MDS pathogenesis. *Conclusions.* We try to point out the role of apoptogenic factors related to TRAIL and the signalling pathways of PKC-epsilon in the pathogenesis and evolution of MDS. This could be useful for supporting novel therapeutically tools aimed to interfere with this signalling pathways.

PO-005

HEMATOPOIETIC CHIMERISM AFTER ALLOGENEIC STEM CELL TRANSPLANTATION: COMPARISON BETWEEN QUANTITATIVE REAL-TIME PCR AND STANDARD METHODS

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Introduction. Increasing mixed chimerism (MC) represents a poor prognostic factor after allogeneic stem cell transplantation (SCT). Moreover, to define the best timing of immune-suppression withdrawal and donor lymphocytes infusion, a strict monitoring of donor hemopoiesis is needed. Methods: We evaluated 18 donor/recipient pairs using a quantitative real-time PCR (qrt-PCR) with the aims 1) to evaluate the informativeness of this chimerism assay and 2) to compare qrt-PCR analysis with standard methods such as fluorescence in situ hybridization (FISH) for mismatched sex pairs or variable nucleotide tandem repeats (VNTR) for matched sex pairs. Qrt-PCR (LightCycler 2.0, Roche) was performed on bone marrow and peripheral blood samples collected monthly, using eleven biallelic DNA genetic system located on chromosomes 1, 6, 9, 11, 17, 18, 20, X and Y. Glyceraldeyde phosphate dehydrogenase (GAPDH) gene was used as active reference. Before quantification, donor and recipient DNAs were genotyped using primers and probes specific for all genetic markers. Patients had a median age of 43.5 years (range 26-70) and were affected by acute leukemia (n=12), or lymphproliferative disorders (n=6). Standard regimen was used in 10 cases, reduced intensity conditioning in 4, while 4 patients underwent an unrelated SCT. Median follow-up of the 18 patients was 16.5 months (range 4.2-34.4). Results: Both qrt-PCR and FISH detected donor/recipient differences in 100% of pairs, while VNTR was not informative in 25% of sex matched pairs. Mixed chimerism was observed in 8/18 patients (44.4%) using qrt-PCR and in 3 of the 16 patients (18.7%) evaluable with standard methods. Overall, 5/18 patients (27.8%) relapsed; before relapse, mixed chimerism was observed in all patients by qrt-PCR and in 3/5 by FISH/VNTR. Qrt-PCR detected mixed chimerism 45 days (range 0-315) earlier than standard methods. In 2 cases in which VNTR was either not informative or not predictive for relapse, the interval between detection of mixed chimerism by qrt-PCR and relapse was 30 and 315 days, respectively, *Conclusions.* chimerism determination using qrt-PCR is more informative than standard methods and may represent an useful tool for the follow up of allogeneic SCT.

PO-006

ENDOTHELIAL PROGENITOR AND CIRCULATING ENDOTHELIAL CELLS IN PATIENTS WITH SYSTEMIC SCLEROSIS (SSC): SHORT-TERM SIMVASTATIN TREATMENT IMPROVES ENDOTHELIAL FUNCTION

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Background. In vitro and *in vivo* studies showed that statins have a role in modulating several pathophysiological processes other than those involved in lipid metobolism. Particularly, statins can improve endothelial function by increasing nitric oxide biosynthesis and bioavailability,

and by promoting angiogenesis directly mobilizing endothelial progenitor cells (EPCs) from bone marrow. Recently, we reported the presence of mature circulating endothelial cells (CECs) in patients with SSc, as a probable result of shedding from affected walls of the blood vessels (Arthritis & Rheumatism, 2004;50:1296). We also found that impaired vascular repair in SSc is potentially related to defective production of EPCs by bone marrow, particularly in the end stages of the disease (Arthritis & Rheumatism, in press). *Aim of the study.* To investigate the hypothesis that statins may improve endothelial function in SSc, we evaluated levels of circulating EPCs, CECs as markers of endothelial injury in SSc patients treated with simvastatin. *Methods.* The study cohort included 20 SSc patients with normal cholesterol levels and 20 hypercholesterolemic control subjects. They received 20 mg/day of simvastatin orally for 8 weeks. Peripheral blood samples were obtained before treatment and at the end of therapy. Five-parameter, 3-color flow cytometry was performed with a FACScan. CECs were defined as CD45 negative, CD31 and P1H12 positive cells, whereas EPCs were identified as CD34 and CD133 positive. *Results.* Simvastatin treatment significantly increased EPCs from the baseline [from 3.6+3.5 cells/mL to 8.3+5.1 cells/mL (p=0.04)] in the hypercolesterolemic group, but failed to improve the EPC levels in the SSc patients [from 3.9+3.06 cells/mL to 2.2+ 5.9 cells/mL(p=0.26)]. CECs' baseline levels were significantly higher in SSc patients and in hypercholesterolemic subjects compared with normal controls [28 and 24 respectively vs 13 cells/mL (p=0.004) and p=0.006)] and at the end of the treatment their counts significantly decreased only in the SSc group (from 28 + 22 to 13 + 20 cells/mL, p=0.03). *Conclusions.* Therapy with simvastatin results in a rapid and significant improvement of parameters of endothelial damage in the SSc group, further suggesting a potential role of statins in the peripheral vascular disease treatment of this disease. The lack of effects on the EPC levels confirm our previous findings on the presence of endothelial stem cell defects in the bone marrow of SSc patients and indicate that potential effectiveness of statin treatment in SSc is not related to increased angiogenesis.

PO-007

COMPARISON BETWEEN HEMATOPOIETIC PROGENITOR CELLS' (HPC) AND CD34+ CELLS' COUNT

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It's known that there is a threshold requirement of $CD34^+$ cells for rapid hematological engraftment after PBSC-transplantation. Flow cytometric count actually provides a quantitative and reproducible method to predict the total apheresis yield of $CD34^+$ cells/Kg, but this technique is expensive and it requires long times and a specialized technical staff. An alternative, simplex, rapid and economical method may be the hematopoietic progenitor cells (HPC) count, by an automatic analyzer, during a common hemocromocytometric examination. 17 healthy PBSC donors were studied in order to evaluate the existence of a correlation between the HPC count and $CD34^+$ cells. They were mobilized with G-CSF and, until the PBSC-collection day, $CD34^+$ cell and HPC counts were monitored. HPC were determined on the basis of the resistance to lysing reagent, the cellular volume and structure (as nuclear size and presence of cytoplasmic granules). At the end of our study, no one significative statistical correlation was founded between HPC and $CD34^+$ count demonstrating that HPC measurement does not represent a direct count of stem cells. On the other hand, some authors have affirmed that HPC count is not affected by PBSC-apheresis collection, suggesting that with this assay a different progenitor population was measured. In conclusion, in our opinion, HPC count alone is actually inadequate for predicting apheresis yield, although a HPC screening (as part of an automated blood count) followed by flow citometric $CD34^+$ cell count, restricted to not yet well established laboratory indications, may be more cost effective than the use of $CD34^+$ cell count alone.

PO-008

GROWTH FACTORS RELEASED BY PLATELET RICH PLASMA (PRP) AND PLATELET COUNT OF BLOOD DONORS

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Thanks to the recognition of the role of platelet growth factors (PDGF platelet derived growth factor, TGF β1 transforming growth factor β 1, EGF epidermal growth factor, IGF I and II insulin like growth factor I and II and VEGF vascular endothelial growth factor) in the processes of tissue repair, platelet gel has recently been introduced into clinical use. In fact, platelets produce, store and release numerous growth factors capable of stimulating the reproduction of mesenchymal stem cells, fibroblasts, osteoblasts and endothelial cells, as well as having a chemotactic activity for macrophages, mononuclear cells and polymorphonuclear cells. Research has, therefore, concentrated on the possibility of supplying a strong regenerative stimulus to tissue by applying very concentrated platelets in situ. In fact the PRP, after activation, releases numerous growth factors (GF) capable to accelerate the tissue regeneration and used for non conventional treatment of ulcers and surgical wounds. The aim of this study was to evaluate the existence of a correlation between age, sex and platelet count (PLT) of blood donors with the detectable amount of GF in PRP. PRP was prepared, separating one unit of whole blood, from 225 healthy donors (162 men and 63 women), aged 19-59 years. In order to prepare the gel, 10 mL of PRP were mixed with 1 mL of calcium gluconate and 1 mL of human thrombin. This mix was incubated at room temperature for 5 minutes. GF concentrations and PLT count were assayed in whole blood and in PRP before activation. The GF were assayed in the PRP before and after activation. The mean PLT count in PRP was 5 times higher than in whole blood (1.545±312 vs. 280±55×10^3 /microL). Some GF showed high concentrations respect basal values: PDGF-AB = 134±56 ng/mL, TGF-β1=172±69 ng/mL and IGF-I=96±29 ng/mL; while other GF were only found in little amount: PDGF-BB=15±8 ng/mL and TGF-β2=0.9±0.5 ng/mL. No influence by donor's sex or age on GF was discovered (except for IGF-1). GF in PRP showed a slight correlation with PLT in PRP (p<0.05), but not with PLT in whole blood (p=0.35). GF in PRP showed substantial variations among studied subjects, but the factors influencing their concentrations aren't still fully known. PLT in PRP showed an initial liner positive relation with GF until to go to a plateau. Nevertheless the standardization of procedures for PRP preparation will allow us to resolve this problem, in fact the immunohematological laboratories will be able to prepare PRP with the desired concentration of platelets in order to btain the desidered clinical results.

PO-009

SHORT COURSE ANTIBIOTIC LOCK FOR THE TREATMENT OF CATHETER-RELATED INFECTIONS IN HAEMATOLOGICAL PATIENTS UNDERGOING CHEMOTHERAPY WITH OR WITHOUT STEM CELLS TRANSPLANTATION

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Introduction. Intravascular catheters (CVC) are commonly used for chemotherapy with or without haematopoietic stem cells transplantation (HSCT). Among CVC related infections (CRI), bloodstream infections (BSI) or localized catheter colonizations (LCC) result frequently in catheter loss and in increased morbidity and mortality. Antibiotic lock (ABL) is recommended for treatment of uncomplicated CRI. The aim of this study was to evaluate the safety and efficacy of a short course (3 days) ABL for treatment of CRI in patients undergoing chemotherapy with or without HSCT in tunneled CVC. *Methods.* 27 consecutive ABL were given to 20 patients, for a total of 26 CRI. 26 ABL were given as first line treatment and 1 as second line ABL in resistant CRI. 15 patients were receiving allogeneic HSCT, 1 patient a sequential auto-allogeneic HSCT and 4 patients chemotherapy. Criteria of non eligibility was com-

plicated CRI. 9 cases had BSI and 17 LCC. 8 CRI were treated in a single-lumen Groshong and 18 in a double-lumen Hichmann CVC. Of the 26 CRI, S. epidermidis was the pathogen in 19, S. haemolyticus in 3, multiple gram positive bacteria, including S. epidermidis in 3 and gram negative in 1. In 23 courses, ABL was preceded by systemic antibiotic therapy; in 4 cases, treatment consisted of ABL only. The median interval between the first positive blood-culture and ABL was 7.5 days (mean 9.6, range 3-27 days). Treatment consisted in Teicoplanin as first line ABL and Vancomycin in case of S. haemolyticus, as second line ABL and in patients resistant to a previous systemic treatment with Teicoplanin. *Results.* Resolution was obtained in 21/26 CRI (81%) with first ABL; of the 5 persistent CRI, 3 were not eligible for a second line ABL, 1 was lost at follow up and 1 had a persistent CRI to a second line ABL. Second infection, as complications, was seen in 2 cases. Re-infection free probability of the 21 successful CRI was 40% and 25% at 100 and 180 days from ABL, respectively. CRI-related removal probability of all enrolled cases was 30% and 40% at 100 and 180 days from ABL and 15% and 33% at 100 and 180 days from therapy, respectively. *Conclusion.* In our experience, short course ABL was safe, efficacy and at lower cost compared with those due to CVC removal or longer course ABL. The possibility to limit the period of CVC inability to few days makes this technique routinely feasible and well tolerated by all patients.

PO-010

MULTILINEAGE RESPONSE TO PEG FILGRASTIM (PEG G-CSF) ALONE IN LOW/INT-1-RISK MYELODYSPLASTIC SYNDROMES

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Background. Myelodysplastic syndromes (MDS) are clonal stem cell diseases characterized by ineffective hematopoiesis, multilineage dysplasia and peripheral cytopenias with normocellular or hypercellular marrow. They represent a heterogeneous group of disorders with a varied spectrum of clinical, morphologic, biologic and genetic characteristics. Anemia is present in approximately 90% of MDS patients at the beginning or during the course of the disease and they often require transfusions. Neutropenia and thrombocytopenia can be present in lower percentages. Clinical trials have shown that recombinant human erythropoietin (rHuEpo), alone or in combination with recombinant human granulocyte colony-stimulating factor (G-CSF), is a useful drug for the treatment of anemia in low and intermediate-1-risk MDS patients.There aren't however clear evidences of responses to G-CSF or PEG G-CSF alone. The wide variation in clinical presentation has confused treatment strategies and hindered the development of new therapies. We report results of good long multilineage response to PEG G-CSF (pegfilgrastim) therapy used alone in patients with low/intermediate-1-risk MDS. *Cases and Methods.* Two patients admitted in our department in 2001; a man (case 1) of 69 and a woman (case 2) of 79 years old with MDS (WHO: refractory cytopenia with multilineage dysplasia). The patients were presenting at the beginning: (case1) Hb 9.8 g/dl, WBC 2.4 $\times 10^9$/L, PLT 54.3$\times 10^9$/L; (case 2) Hb 7.9 g/dL, WBC 3.5$\times 10^9$/L, PLT 30.0$\times 10^9$/L respectivily. We have cytogenetic and molecular biology study of the second case: cariotype 46, XX ; policlonal HUMARA. Both started rHuEpo therapy with a good response wich lasted three years (increases in Hb levels > 2 g/dL). When the patients were unresponsive to rHuEpo was required high-intensity transfusion regiment (2-3 blood unit/month. They started immunosoppressive therapy and corticosteroids without benefit. When leucopenia worsened with neutropenia(N <1.0$\times 10^9$/L), therapy with pegfilgrastim alone was started with a 6 mg s.c. dosage every 21 days. *Results*. Both the patients obtained complete hematological recovery after 3 weeks from the first dose. In fact they have shown a normal neutrophils count (N > 2.0$\times 10^9$/L), significant and stable increase in Hb levels (Hb > 8.5 g/dL) without transfusion requirements for one year and only a transitory improvement of platelet count (PLT > 80.0$\times 10^9$/L) for five months. The response to PEG G-CSF was still associated with a relevant improvement of quality of life (QoL). CONCLUSIONS: Our report suggests that PEG G-CSF alone could lead the proliferation and maturation of all hematopietic precursors in selected patients.The structure of PEG G-CSF could affect the receptors of an uncommitted progenitor cell. It could play a role in the redistribution of growth factor receptors on the dysplastic clone. A lot of study *in vitro* and *in vivo* is still necessary for explaining these new clinical evidences.

Hemostasis and Thrombosis

PO-011

RITUXIMAB AND THROMBOTIC THROMBOCYTOPENIC PURPURA: OUR EXPERIENCE

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Thrombotic thrombocytopenic purpura (TTP) is a rare disorder usually caused by autoantibody inhibitors of VWF (von Willebrand factor) cleaving protease (ADAMTS-13). Plasma exchange (PEX) is the standard treatment for TTP but the value of additional therapies remains unclear. Treatment options are limited and, especially for refractory patients, often unsuccessful. Rituximab, a chimeric monoclonal antibody against CD20, has been recognized as a useful therapy for antibody mediated autoimmune diseases. Rituximab binds to CD20 positive B cells and depletes them via antibody dependent cellular cytotoxicity (ADCC), inducing apoptosis and complement mediated lysis. We usually treat our patients, according to GIPTT (Italian Group Thrombotic Thrombocytopenic Purpura), with seven PEX in nine days, methylprednisolone (1 mg/Kg/die) and acetylsalicylic acid. Responder patients (Platelets >100$\times 10^9$/L since at last two days and stable or improved neurological condition) then undergo three more PEX in one week followed by two PEX in one week with methylprednisolone gradually reduced till suspension one month after diagnosis. Not or partially responders ones (Platelet count <50$\times 10^9$/L or 50$\times 10^9$/L<Platelet<100 $\times 10^9$/L) are treated with methylprednisolone (1 mg/Kg/die), five PEX, using cryosupernatant as the replacement fluid, four doses of Rituximab in one week and optionally two doses of vincristine, before a revaluation: if they can be now considered as responders two more PEX in one week are administered with a dose of Rituximab after 15, 30, 60, 90, 150 and 210 days. During the last two years we treated three women with severe acute TTP after they failed standard therapies with Rituximab according to the described protocol. Two patients received three weekly and three monthly doses (2 mg) of vincristine too. Two patients responded with prompt improvement in microangiopathic haemolytic anemia, achieved augmentation of their platelets counts and reached a complete remission; they are now healthy. The third patient's conditions got better but she died of ARDS (Acute Respiratory Distress Syndrome). All patients showed evidence of response to anti CD20 antibody. It is our opinion that rituximab therapy should be considered as a useful immunomodulating adjunct in the treatment of refractory and relapsing TTP.

PO-012

ANTI-CD20 MONOCLONAL ANTIBODY (RITUXIMAB) IN THE TREATMENT OF CHRONIC IMMUNE THROMBOCYTOPENIC PURPORA

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In recent years, clinical studies have been undertaken with selected monoclonal antibodies (MoAbs) in the treatment of several haematological disease, especially in malignant disorders. However, clinical observations indicate that MoAbs may be an important alternative for the conventional therapy of some autoimmune disorders. Autoimmune cytopenias have been investigated in the last few years with positive preliminary results. Rituximab (MoAb directed against CD20 antigen) seems to be an effective and safe agent for the treatment of immune thrombocytopenia, autoimmune hemolytic anemia, cold agglutinin disease and pure red cell aplasia. This study assesses the efficacy and safety of Rituximab in 12 adults with chronic immune thrombocytopenic purpora (ITP). The patients were resistant to previous two or more ITP treatments and all had undergone splenectomy. Rituximab was administered at a dose of 375 mg/m^2 once weekly for a total of four infusions for the initial course, as well as re-treatment. A complete response (CR) was defined as an increase of platelets to normal counts (>150$\times 10^9$/L), a partial response (PR) as an increase to between 50 and 150$\times 10^9$/L. All patients responded, achieving a platelet count >50$\times 10^9$/L: 7 achieved a CR and 5 a PR. The response occurred within 8 weeks of the first infusion. Duration of response was considered from the day of the initial

infusion to the first time of relapse (platelet count <30×10^9/L). All 7 CR patients continued in CR after more than 1 year from the first infusion of Rituximab with no additional ITP treatment. 3 of 5 patients who achieved a PR still had counts >50×10^9/L at 48 weeks from treatment, the remaining 2 PR patients relapsed at a median of 10 weeks. Since then, the 2 patients were successfully retreated with a second complete response, at the time, of 6 months duration. No patients had adverse events related to Rituximab infusion. Circulating CD19 positive B cells fell to <0.03×10^9/L within 4 weeks and recovery of circulating cells began between weeks 12-14. No changes in immunoglobulin levels or infection complications were seen. In summary, Rituximab was well tolerated with no immediate complications and induced a lasting, substantial good response. Although the case series are small, Rituximab seems to be an effective and safe agent for the treatment of this disease, however, a longer follow-up and the studies on larger number of patients are needed to determine the real value of these new approaches in autoimmune cytopenia.

PO-013

A TYPE 2B VON WILLEBRAND DISEASE (VWD) PATIENT WITH A FULL SET OF MULTIMERS LINKED TO THREE MUTATIONS (V1229G, N1231T, P1266L) IN THE A1 VON WILLEBRAND FACTOR (VWF) DOMAIN AND A MILD PLATELETS SECRETION DEFECT

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The patient was a 26 years-old woman when she required transfusion because of severe bleeding after abdominal surgery. She was found to have normal/borderline VWF activities (VWF:Ag 64%, VWF:RCo 54%, VWF:CB 57%) with an enhanced affinity for the platelet glycoprotein Ib α (0.6 mg/mL RIPA) and a borderline/normal platelet count (103-172 ×10^9/L). Despite a normal VWF multimeric pattern in her plasma, she was found to carry 5 distinct nucleotide substitutions (3686T>G, 3692A>C, 3735G>A, 3789G>A and 3797C>T) on the VWF exon 28 encoding for the A1 domain. Two of these substitutions were silent, whereas the remaining caused the following amino acid changes: V1229G, N1231T and P1266L. The mutations were found also in her father, paternal grandfather and uncle confirming that all defects were carried by a single allele. The three missense mutations have already been reported: V1229G, N1231T together were found in a patient classified as type 1 (Thromb Haemost 1998;79:709), whereas P1266L was identified in the type 2B New York and Malmo (J Clin Invest 1993;91:77). The fact that all five substitutions found in this patient correspond to the pseudogene sequence, sustain the possibility of a gene conversion between the VWF gene and pseudogene, as reported by others (Thromb Haemost 1998, 79:709 Blood 2001;98:248). The patient was the only one among other three family members with the same genetic defect who presented bleeding symptoms. In some patients with type 2B Malmo, additional risk factors for bleeding such as impaired collagen-induced platelet aggregation have been reported (J Thromb Haemost 2004;2:2055). Further investigation performed in our laboratory, indeed showed a mild platelet secretion defect when platelets were stimulated with ADP. Our data confirm that VWF defects can be complex within VWD families and that bleeding tendency can vary according to additional defects of haemostasis.

PO-014

HOME THERAPY FOR DEEP VEIN THROMBOSIS AND PULMONARY EMBOLISM IN CANCER PATIENTS. LA TERAPIA DOMICILIARE NEI PAZIENTI ONCOLOGICI AFFETTI DA TROMBOSI VENOSA PROFONDA E/O EMBOLIA POLMONARE

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Background. Outpatient treatment of deep vein thrombosis (DVT) has become a common practice in uncomplicated patients. Scanty data are presents in patients with comorbidity (such as cancer) or concomitant symptomatic pulmonary embolism (PE). Cancer patients with DVT are often excluded from home treatment because of high risk of bleeding and recurrent DVT. We tested the feasibility and safety of the Home Treatment (HT) program in cancer patients with acute Venous Thromboembolism (VTE). *Material and Methods.* Consecutive cancer patients having a confirmed episode of DVT or PE were treated as outpatients unless they required admission for other medical problems, were actively bleeding or had pain requiring parenteral narcotics. Anticoagulant treatment for VTE was based on Low Molecular Weight Heparin (LMWH) followed by warfarin or LMWH alone at therapeutic dosages. An educational program for patients was implemented during the index visit. Results. Over a period of 3 years, 207 patients with cancer and acute VTE (139 with DVT and 68 with PE) were evaluated; 36 (17.4%) of them had metastatic disease. Treatment with LMWH and warfarin was prescribed to 106 (51.2%) while LMWH alone to 102 (48.8%). One hundred and twenty-seven patients (61.3%) (91 with DVT and 36 with PE) were entirely treated at home. Reasons for hospital admission in the remaining patients (n. 80) were poor compliance [22, (27.5%)], concomitant serious illness [52 (65%)] and refusal of home-treatment [6 (7.5%)]. There were no differences between patients treated at home and those hospitalized with regard to gender, mean age, site of cancer, presence of metastases and choice of anticoagulants (Table). After 6 months, recurrent DVT, PE and major bleeding occurred in 6.5%, 5.5.% and 1.5% of patients treated at home, and 8.3%, 9.3% and 2% of those hospitalised. These differences were not statistically significant (p=0.58). Twenty-seven patients (33%) in the hospitalized group and 33 (26%) in the home-treatment group died as a consequence of neoplasm. Conclusions. These results indicate that, regarding cancer patients with acute DVT and/or PE, there is no difference between hospitalised and home-treated patients in terms of major outcomes.

Table. Complications occured in Low- and High-Risk Groups.

	Standard in hospital		*Home Therapy*		*p value*
Number of patients	48 DVT	32 PE	91 DVT	36PE	n.s.
Mean age (range)	68.6 (37-92)		61.5 (32-90)		n.s.
Males	45 (56.2%)		67 (52.7%)		n.s.
Proximal DVT	43 (89.5%)	6 (18.7%)	82 (90.1%)	7 (19.4%)	n.s.
Distal isolated DVT	5 (10.4%)	2 (6.2%)	9 (9.8%)	2 (5.5%)	n.s.
Symptoms of PE*	7 (12.5%)	-	8 (8.7%)	-	n.s.
Metastatic cancer	10 (20.8%)	7 (21.8%)	13 (14.2%)	6 (16.6%)	n.s.
Site of cancer, n (%)					
Gastrointestinal	33 (41.2)		56 (44.1)		
Genitourinary	21 (26.2)		26 (20.4)		
Breat	26 (32.5)		24 (18.9)		n.s.
Lung	6 (7.5)		14 (11)		
Haematologic	4 (5)		7 (5.5)		
Mean time from cancer to VTE diagnosis	30.8 months		28.9 months		n.s.
Ongoing chemo-, or radio or hormone therapy, n (%)	32 (40)		43 (33.8)		n.s.
Co-morbidity	21 (43.7%)	21 (65.6%)	55 (60.4%)	20 (55.5)	n.s.
In-hospital stay	8±2 days		3.1 hours		<0.0004

** In DVT patients only*

PO-015

THE VON WILLEBRAND DISEASE (VWD) TYPE 2A (II H): A UNIQUE VARIANT OF VON WILLEBRAND FACTOR (VWF) LINKED TO 3 DISTINCT MUTATIONS

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Type IIH VWD, classified now as type 2A, is a unique VWF variant that has been shown to have abnormalities different from other variants previously described (IIC, IID, IIE, IIF and IIG), i.e. the absence of high molecular weight (HMW) VWF in plasma and platelets and the absence of the triplet structure on high resolution agarose (1.6% LGT) gel. The patient is a man who was 30-year-old at the time of diagnosis, with a life long bleeding history (epistaxis, ecchymosis and prolonged bleeding after dental extractions). Although, 5 additional family members were investigated (Am J of Hematol 32:287, 1989), only the propositus showed bleeding symptoms, suggesting a recessive inheritance of the disease.

SSCP analysis was performed evaluating all exons of the VWF gene. Three distinct novel mutations, not identified in 100 normal chromosomes, were found: 604 C>T (R202W), 2546 G>A (C849Y) and 2546 G>A (R1583Q). Mutations R202W and R1583Q were found to be on the same allele, since were both identified in 2 propositus' relatives. The absence of HMW VWF in the propositus' platelets suggests a multimerization defect that could be due to mutations R202W (D1 domain) and C849Y (D' domain). In fact, both domains are involved in the multimerization process. However, the VWF multimerization is compromised only in the presence of both defects, since carriers of R202W present a normal multimeric pattern. The absence of a triplet structure, was confirmed in the propositus, his father and his daughter and is perhaps linked to R1583Q mutation. The presence of a mutation C849Y in the D' domain prompt us to investigate the VWF-Factor VIII binding in the propositus, that behaved similarly to a known type 2N heterozygous variant. Future expression studies maybe able to correlate the mutations to the patient phenotype and explain the absence of the triplet structure.

PO-016

IMPAIRED COLLAGEN BINDING ACTIVITY (VWF:CB) OF VON WILLEBRAND DISEASE (VWD) TYPE 2B

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Among the different tests used for VWD diagnosis, VWF:CB has been proposed as additional test to further characterize different VWD variants, despite the fact that has not been standardized yet. In our laboratory we perform VWF:CB with collagen from two different sources: VWF.CB(I) (equine type I from Nycomed) and VWF:CB(III)(human type III from Southern Biothecnologies). Among 83 samples from different VWD patients and 22 normal controls, we found that in VWD type 2B patients the VWF:CB(I) was significantly more reduced than VWF:RCo. In all VWD 2B patients the VWF:CB(I) levels were lower than VWF:RCo, even in patients with normal VWF multimers. The VWF:CB (III) behaved differently and appeared to reflect only the loss high molecular weight multimers.(HMWM) The VWF:CB (I)/Ag ratios of patients with type 2A and 2B VWD with abnormal multimers were very similar, even though the VWF multimeric structure of 2A is more defective than that of type 2B VWD patients, whereas the VWF:RCo/Ag ratios were clearly different. These data suggest that type 2B VWF variants might also have a reduced VWF:CB not only related to the loss of HMWM.

Table.

VWD Type (n)	*VWF:RCo/Ag*	*VWF:CB(I)Ag*	*VWF:CB(III)/Ag*	*RIPA mg/mL*
N (22)	0.97±0.18	1.04±0.2	1.03±0.2	1.00±0.1
1 (20)	0.96±0.2	1.09±0.3	0.99±0.1	1.3±0.15
2M (20)	0.20±0.1	0.80±0.2	0.82±0.1	1.7±0.2
2A (14)	0.17±0.1	0.12±0.1	0.38±0.2	2.1±0.7
2B (21)*	0.48±0.2	0.13±0.1	0.58±0.3	0.59±0.13
2B (8)**	0.66±0.2	0.5±0.1	0.75±0.2	0.47±0.13

** Abnormal multimers ** Normal multimers*

Hematologic Diseases (non-Oncologic): Molecular Basis

PO-017

ROLE OF HUMAN CD38 IN B CELL SIGNALING

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Human CD38 is a multifunctional molecule displaying ecto-enzymatic and receptor activities. CD38 molecules on the cell surfaces are known to be part of large supramolecular complexes in lymphoid and myeloid compartments. CD38 is functionally dependent on its partners, as shown for the TCR in T cells and for CD16 in NK cells. The aim of this work is to study the membrane localization, lateral associations and signaling properties of CD38 in human B lymphocytes. Membrane fractionation showed that a relevant amount of CD38 molecules (~60-70%) is constitutively present within detergent insoluble component of the membrane in a panel of B cell lines, corresponding to different development stages (Nalm-6, Daudi, Raji and RPMI-8226) and in normal tonsil B lymphocytes. CD38 ligation by means of agonistic monoclonal antibodies induces translocation of all molecules into the lipid rafts, suggesting that membrane compartimentalization of CD38 is a way of functional regulation. CD38 cross-linking also induces a physical association with CD19, as detected by co-immunoprecipitation and co-capping experiments. The association seems to take part predominantly within the rafts, as confirmed by its loss in the presence of methyl-β-cyclo dextrin (MβCD), which is responsible for partial cholesterol depletion. CD38 association with CD19 is also functional: CD38-mediated Ca^{2+} fluxes are only apparent when CD19 is present and active, while they seem to be independent of BCR. These data are also confirmed using siRNA to silence CD19 expression. These results suggest that: i) CD38 is a signaling molecule in B cells, localized in high percentage within the rafts; ii) it associates with CD19 after receptorial engagement, to transduce its signals.

PO-018

POLYMORPHISMS AND HAPLOTYPES IN FOLATE-METABOLIZING GENES DO NOT EXPLAIN THE CLINICAL MANIFESTATION OF COBALAMIN DEFICIENCY

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Introduction. Vitamin B12 deficiency is common and its most typical cause is believed to be food B12 malabsorption caused by chronic gastritis, gastric atrophy, and the use of proton pump inhibitors. The classic pathophysiological manifestations of B12 deficiency include megaloblastic anemia and neurological complications. Methyltetrahydrofolatereductase (MTHFR) plays a central role in directing in directing the folate pool toward the remethylation of homocysteine to methionine at the expense of purine and DNA synthesis, and it is known that B12 deficiency impairs overall folate metabolism because 5-methyltetrahydrofolate (5MTHF) becames metabolically trapped. The 677C →T and 1298A →C polymorphisms of the MTHFR gene are associated with mildly decreased MTHFR activity. Matherial and methods. We examined whether the cobalamin deficient patients with reduced MTHFR activity were predisposed to neurological dysfunction and/or to have anemia. We studied 23 patients with megaloblastic anaemia, that included 13 men and 10 women, with a median age of 55 years (range 24-85). At diagnosis, hemoglobin (Hgb) median value was 7 g/dL (range 3.5-12.6, normal values 14-18), white blood count (WBC) median value was 4170/mm^3 (range 1160-7740, normal values 4100-9800), medium cellular volume (MCV) median value was 111 fL (range 87.1-138, normal values 81-99), plasmatic B12 median value was 43 pg/mL (range 15-94, normal values 158-600 pg/mL), and plasmatic folates median value was 6.2 ng/mL (range 0.9-24.4, normal values 3-15 ng/mL). MTHFR C677T and A1298C genotypes were analyzed on DNA from peripheral blood cells according to Frosst and Weisberg method. *Results.* We found the MTHFR 677 CC genotype in 13 cases (56%), the 677CT in 6 cases (26%), and the 677TT in 4 cases (17%). For MTHFR 1298, we observed the 1298AA in 9 cases (39%), the 1298AC in 12 cases (52%), and the 1298CC in 2 cases (8%). Univariate analysis including Hgb, WBC, MCV,

B12 and folates levels at diagnosis with respect to genotype did not show any significant difference in 677 and 1298 genotypes distribution. We then explored possible correlations between different genotypes and neurological manifestations and gastric atrophy (evidenced with gastric biopsy), and we found a significance correlation between gastric atrophy and the 677TT genotype (p=0.004), while the same datuum wasn't present for the 1298CC genotype; no correlations were evidenced for what reguards neurological symptoms and the different MTHFR genotypes. *Conclusion.* Our results show that the two MTHFR polymorphisms, which help to shunt folate towards thymidilate syntesis and away from methionine synthesis, neither protect against anaemia nor predispose to neurological dysfunction in patients who develop cobalamin deficiency. It has been suggested that the MTHFR 677TT genotype may protect patients with low methionine synthase activity from DNA biosynthesis defects because folate metabolism in these patients tends to be diverted to the synthesis of formylated folates rather than 5MTHF. We found a correlation between MTHFR 677TT and a state of autoimmune disorder such as atrophic gastritis. Other studies must be sought for the explanation of this association.

PO-019

N-TERMINAL PRO-BRAIN-NATRIURETIC-PEPTIDE (NT-PROBNP) LEVELS ARE INCREASED IN SICKLE CELL DISEASE PATIENTS FREED FROM PULMONARY HYPERTENSION

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Sickle cell disease (SCD) is one of the most common monogenic disorders and it is related to the presence of the mutated hemoglobin S (HbS). The clinical manifestations of SCD are the chronic hemolytic anemia and the recurrent vaso-occlusive crises, which are responsible for acute and chronic organ damage. SCD has been proposed to be a chronic inflammatory disease, associated with abnormal vascular endothelial activation and endothelial damage, which participate to generation of SCD vaso-occlusive crisis. Recently, the tissue ischemic/reperfusion injury, such as during brain stroke or coronary heart disease, has been associated with increase of N-terminal pro-brain natriuretic peptide (NT-proBNP) in response to hypoxic stimulus. In addition, high levels of BNP levels have been shown to increase in SCD complicated by pulmonary hypertension (PH). Thus, we asked whether NT-proBNP might be affected by sickle cell disease before the appearance of clinical evidences of PH, in relation to chronic SCD vascular endothelial damage. We studied 28 SCD patients freed from PH, evaluated by echocardiography; 25 were Africans and 3 were Caucasians. The patients were divided as follow: 17 SS, 5 SC, 4 βS and 2 AS patients. Patients' age ranged between 18 and 45 years-old. A corresponding population of normal controls matched for age, sex and race was used as reference group. In steady state and under acute mild vaso-occlusive crises (n=5; VOC-episodes), defined by visual analogue scale for pain, we measured the following parameters: NT-proBNP, cardiac Troponin T (cTnT), ischemic modified albumin (IMA), C-reactive –protein (CRP) complete blood count, HbS level, creatinine, LDH, albumine. Age and sex-adjusted diagnostic thresholds for cTnT, IMA and NT-proBNP were <0.01 ng/mL, <85 Kunits/l and 125 pg/mL, respectively. Total imprecision, as expressed by the coefficient of variation, was comprised between 3 and 6% for IMA, creatinine and albumin and lower than 10% for NT-proBNP and cTnT. Results of measurements and relative values distributions between patients and healthy matched controls were compared by Mann-Whitney test. In steady state SCD patients showed significant increased of NT-proBNP and IMA levels compared to normal controls, CRP was slightly increased, whereas cTnT, creatinine, albumine were within the control range. We did not find any correlation with age, creatinine, LDH levels; whereas, NT-proBNP indirectly correlate with hemoglobin levels. In the 5 acute VOCs, no significant changes in NT-proBNP or cTNT were evident compared to steady state values. These data indicate that NT-proBNP increases in SCD patients before development of PH and suggest that NT-proBNP might be related to the chronic vascular endothelial injury and associated ischemic/reperfusion damage.

PO-020

OCCURRENCE AND DISTRIBUTION OF VHL-DEPENDENT POLYCYTHEMIA IN SOUTH ITALY

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The Chuvash form of familial polycythemia (MIM 263400) is an autosomal recessive disorder due to Von Hippel Lindau (VHL) gene mutation (Arg200Trp) leading to excessive erythrocyte production in response to normal O2 blood level. This disease, characterized by increased thrombotic and cardiovascular risk, is endemic in Chuvashia where it probably arose 14000-60000 years ago. This disorder was initially described in more than 100 individuals from 80 families living in the mid-Volga river region of European Russia. The mutation was also identified in 4 additional families of non Chuvash origin. Finally, 9 other VHL mutations (Pro192Ala, Hys191Asp, Leu188Val, Tyr175Cys, Gly104Arg, Val130Leu, Asp126Tyr, GÍy104Val and Arg79Cys,) were also detected in single polycythemic patients. VHL participates in the hypoxia-sensing pathway, as it binds the proline-hydroxylated form of the hypoxia inducible factor1-α (HIF1-α), catalyzing its ubiquitination and committing the protein for proteasomal degradation. Under normoxic conditions an active hydroxylation of HIF-1 α allows VHL to down-regulate the transcription factor. Conversely, VHL functional alterations result into HIF complex increase causing overexpression of its target genes, including Epo, VEGF, transferrin, transferrin receptor and several others. The high Epo serum level has been suggested as the major cause of polycythemia; however, the premature mortality of Chuvash polycytemic patients, mainly due to cerebral vascular events and peripheral thrombosis, may be due to mechanisms other than hyperviscosity and serum Epo level. In this study, we investigated 28 South Italian patients belonging to 18 families, presenting with a Chuvash-like polycythemia (elevated Hb level, inappropriately high serum Epo level, normal leukocyte and platelet counts). The analysis of 2 independent families from Ischia (Naples), which included 10 polycythemic cases, surprisingly showed subjects homozygous for the typical Chuvash mutation (VHL598C>T) who had one of the parents in homozygote condition. Since the other parent was heterozygous for the mutation and not consanguinous with other members of the families, we inferred that the VHL598C>T mutation must have a very high frequency in Ischia. Therefore, we evaluated the mutated allele frequency in the island, demonstrating that the mutation is endemic in Ischia, with a frequency (0.07) higher than that in Chuvashia. The haplotype of all patients was identical to that observed in Chuvashia, supporting the hypothesis of a single founder. Conversely, when we investigated 60 healthy subjects from other Italian regions, we were unable to detect any VHL mutation. We also identified 3 polycythemic patients (2 families) from Puglia and Sicily who had high Epo serum level. The patients resulted double heterozygotes, showing a classical Arg200Trp mutation combined with two novel alterations, namely Leu89Phe (265C>T transition) and Pro146Arg (437C>G transition). It has to be underlined that both the heterozygote parents with the Arg200Trp VHL mutation were from Ischia neighboring, confirming the high frequency of the mutation in this area. Finally, we identified other 4 polycythemic patients (4 families) from Campania and Calabria with high Epo serum level. Intriguingly, all these cases showed the VHL mutation in heterozygosity. In conclusion, we have identified a large cluster of Chuvash polycythemia out of Chuvashia, suggesting that this familial erythrocytosis might be endemic in other regions of the world. In addition, we have evidenced the occurrence in South Italy of novel VHL mutations involved in the development of Chuvash-like erythrocytosis.

PO-021

RELEVANCE OF A ROUTINE AUTOMATED SCREENING FOR PRESENCE OF SCHISTOCYTES

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Introduction. Fragmented red blood cells (FRCs) or schistocytes are produced in peripheral blood (PB) after a mechanical damage of erythrocyte membrane. The cells damaged take the characteristic triangular or helmet-shaped morphology. The most frequent cause of erythrocyte frag-

mentation is the cutting of RBC within the microthrombi in the setting of thrombotic microangiopathies such as thrombotic thrombocytopenic purpura (TTP), haemolytic-uremic syndrome (HUS) and disseminated intravascular coagulation (DIC). Schistocytes could be also produced after surgery, bone marrow transplantation, in HIV infection as well as in presence of vessel abnormalities. The persistence of FRCs in peripheral blood produces microspherocytes. Schistocytes are usually evaluated on PB smears by optical microscope observation and are estimated as percentage of total red blood cell (RBC), counting 10,000 erythrocytes. Normal values are as follow: <0,1% of RBC in adults, 0, 3-1,9% in newborn and <5,5% in prematures. The enumerating of FRCs is now available in automation by ADVIA 2120 (Bayer-Tarrytown NY) and XE-2100 (Sysmex corporation, Kobe, Japan) hematology analyzers. The aim of our study is to evaluate the precision and accuracy of automated count of schistocytes using ADVIA 2120 and XE-2100 hematology instruments versus the manual count as the reference method. *Matherials and method.* 204 K3EDTA-anticoagulated peripheral blood samples were analysed from 169 subjects, 88 females, 81 males, median age 48 years (0-95). Samples derived from patients suffering from haematological (anaemia, acute myeloid leukaemia, TTP, lymphoproliferative diseases), cardiac, kidney (acute or chronic renal failure), neoplastic, liver, obstetric and infectious diseases, preterm newborns and patients with a specific clinical query for schistocytes. Moreover samples from the routine work, which were positive for at least one instrument, were included. Schistocyte optical count is done on May-Grunwald-Giemsa stained PB smears on 10,000 RBCs. The identification and the enumeration of FRCs with the ADVIA 2120 method is performed by integrated analysis of RBC and platelet count: they correspond to events with a volume smaller than 30 fentoliters and with a refractive index greater than 1.4, with a frequence of events above the threshold of 10,000/microliters. In Sysmex XE 2100 RET channel, the whole blood, stained with a fluorescent dye specific for RNA/DNA, is analyzed by flow cytometry with a high power argon laser. The FRCs are identified on the scattergram on the basis of intensity of forward scatter and fluorescence. The reference positive value for schistocytes in the XE-2100 system is >0,2%. *Results.* The overall correlation coefficient of the analyzers count versus the manual reference method is R^2 0.5449 and $R^{20.5423}$ for ADVIA 2120 and XE-2100 respectively. Analysing separately the mean two groups of positive samples from patients from surgical and intensive care unit (ICU) the correlation between manual and automated count highly improved with an R^2 0, 9945 and R^2 0, 9257 for ADVIA 2120 and XE-2100 respectively. The range values of positive samples were as follow: reference method :0,1-2,3%, ADVIA 2120 method: 0,2-4,13%, XE-2100 method: 0,5-8,39%. Assuming the value >0,2% from XE-2100 to discriminate positive samples, ROC curve produces a sensibility of 91,1% and a specificity of 66,2%. *Discussion.* In our study no false negatives are found despite the heterogeneous population examined. Adopting the reference value analysers-related versus the reference optical method, no false positive samples are found. In positive samples automated count of FRCs strictly correlates to optically schistocyte count. We conclude that the automated availability of RCFs enumeration is very useful in clinical practise offering the possibility to obtain a accurate and precise count of positive samples at diagnosis and during follow-up.

PO-022

CA 15-3 A MARKER FOR THE MEGALOBLASTIC ANAEMIA?

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Ca 15-3 is a glycoprotein present in the cells of the mammary carcinoma and in some epithelial cells. Is a marker used for the monitoring of the breast and gastrointestinal carcinoma. The megaloblastic anaemia is an anaemia characterized by deficit of absorption of Vitamin B12 and is associated with gastritis atrophic and the target cell is the parietal gastric cell. In our institution, from June 2003 to February 2006, the level of Ca15-3 and of others tumour markers (CEA; Ca125; Ca19.9; α-FETO) they have been tested in the serum of 22 patients (14 male and 8 female with median age of 60 and range of 36-80 years) with de novo megaloblastic anaemia, 3 patients were gastrectomized. In all patients has been effected: esophagogastroduodenoscopy and control anti-parietal gastric cells antibody (APCA). Increase level in the serum of CA 15-3 with normal level of other tumour markers have been found in 19/22 patient with median value of 61 U/mL (range 35-100 U/mL) in 3 patients (gastrectomized) the value of CA 15-3 was normal. Besides in 17/19 patients have shown positivity for the APCA, only in two patients has been diagnosed a gastritis atrophic, in the other patients has been observed a normal gastric mucous. After a median observation of 36 months any patient has developed a mammary or gastro-intestinal carcinoma. These results indicate what the increased level of CA 15-3 antigen in patient with megaloblastic anaemia is positively correlated with APCA and with the presence of a normal gastric mucous. These clinical conditions make to suppose the destruction from the APCA of the parietal gastric cells with the liberation of this glycoprotein and this is shown in the 3 patients gastrectomized with presence of APCA and not increased level of the CA 15-3. In conclusion the CA15-3 antigen is probably an specific marker for the diagnosis of megaloblastic anaemia and is probably associated with the destruction of parietal gastric cells.

PO-023

LEPTIN AND ITS RELATIONSHIPS WITH BLOOD PARAMETERS IN VARIOUS HEMATOLOGICAL DISEASES

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Introduction. Leptin, secreted from white adipocytes, is a 16 kDa protein that exhibits a functional pleiotropy mediated by leptin receptor (LR). In rodentes and in human, it seems to be implicated in the regulation of food intake, energy expenditure and basal metabolism. Its primary physiologic role consists in controlling the adipose tissue metabolism, but it also play a role in fetal and adult erythroid and myeloid development. Moreover the presence of the LR on $CD34^+$ cells and the proliferative effects on haematopoietic cells in culture suggest a link between leptin and haematopoiesis. The aim of this study was just to evaluate an eventual existence of a relationship between leptin and haematological parameters in various hematological patients. *Methods.* We studied 278 hematological patients; in particular, 51 were affected with sideropenic anemia, 18 with idiopathic thrombocytopenia, 27 with β thalassemia major, 11 with hereditary hemochromatosis, 34 with polycythemia vera, 26 with aplastic anemia, 51 patients with multiple myeloma, 60 with monoclonal gammopathy of undetermined significance. 151 healthy non-obese blood donors (89 men and 62 women) were also evaluated as control group (CG). Hemocromocitometric examination and determination of serum leptin levels were performed at the time of diagnosis for patients and of blood donation for CG. The relationships of serum leptin levels with Erythropoietin (Epo), Thrombopoietin (Tpo) and blood cell parameters were evaluated. *Results.* Serum Leptin levels shown a wide range of oscillation in patients (0.07-147 ng/mL) respect to the controls (0.1-16 ng/mL) and a concentration >20 ng/mL was observed in the 8% of patients; nevertheless our results demonstrated no statistically significant differences between the two groups (mean±Standard Deviation = 13.1±14.5 ng/mL for patients versus 12.0±14.8 ng/mL for CG). Leptin in all groups was positively correlated with the body mass index (BMI). In both populations, leptin concentration was significantly higher in women than in men, even if compared for body mass index (BMI). Leptin was positively correlated with leukocyte count in patients, while no correlation was found in controls. Correlations between leptin and erythrocyte count, hematocrit, hemoglobin, platelet count, Epo, Tpo or other parameters were not found. *Conclusion*. High leptin concentrations are reported in various diseases, such as renal failure and sepsis. Leptin secretion is also influenced by drugs, such dexamethasone, adrenoceptor agonists and insulin. In this study, no significant relationships were found between leptin and erythropoietin, thrombopoietin or other blood parameters. Moreover no correlation was found between leptin and clinical status or therapy, although many patients were suffering from a cronic illness. However, a role for high concentrations of leptin in critical hematological situations cannot be excluded and, in these conditions, leptin could contribute, with other cytokines, to the fine regulation of hematopoiesis.

PO-024

DEFORMABILITY-BASED *IN VITRO* ANALYSIS OF RED BLOOD CELLS FLOWING IN MICRO-CAPILLARIES

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The deformability of red blood cells flowing in microvessels is essential to maintain optimal blood circulation and to allow gas transfer between blood and tissues. Reduced RBC deformability is involved in a number of blood dissorders, such as Thalassemia, Iron Deficiency, Congenital Spherocytic and Non Spherocytic Anemias, Idiopathic Myelofibrosis. In spite of its physiopathological relevance, measurements of RBC deformability are usually of difficult clinical application, being still carried out by approximate methods and under conditions quite different from those occurring *in vivo*. We have started investigating the deformability of RBCs flowing in microcapillaries made from silica or drawn in a gel matrix. The internal diameter of the microcapillaries used in this work ranges from 6.5 to 2 micron, i.e., close to the average size of RBC or even smaller. The microcapillaries are placed in a rectangular flow cell, where a suspension of RBCs, isolated by centrifugation and properly diluted in albumin-additioned ACD, is fed through a syringe under the action of a liquid head in the physiological range. The flow cell is mounted on a motorized x-y stage of an inverted microscope equipped with a CCD camera. High magnification images of RBCs under flow are aquired at short exposure times by using an electronic shutter, digitized by a frame grabber, and stored on hard disk for later analysis to measure type and extent of cell deformation. Experimental variables include flow rate, size and length of microcapillaries. RBCs from healthy donors and from patients with various haematological disorders have been investigated; preliminary results are presented and discussed.

PO-025

ERK5 MEDIATES THE MACROPHAGE COLONY-STIMULATING FACTOR SIGNALLING IN A SRC-DEPENDENT AND PKC- INDEPENDENT FASHION

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The Macrophage Colony-Stimulating factor (M-CSF) sustains the survival and proliferation as well cell spreading and adhesion of monocyte/macrophage cells. ERK5 (also known as big MAPK1-BMK1) a member of the mitogen-activated protein kinase (MAPK) family, is a 98-kDa molecule sharing homology with ERK1/2. ERK5 is activated by a kinase cascade involving the upstream kinases MEK5, MEKK2/3 and Tpl-2. ERK5 plays a role as a redox-sensitive protein kinase, and may be activated in response to growth factor stimulation. This study was undertaken to characterize the involvement of ERK5 in M-CSF elicited signaling in the murine macrophage cell line BAC1.2F5 and in human bone marrow derived macrophages. Exposure to M-CSF resulted in a rapid and transient induction of ERK5 phosphorylation in activation-specific residues. Moreover, upon exposure to M-CSF, ERK5 showed a rapid and transient translocation from the cytosol to the nucleus, as indicated by immunofluorescence and cell fractionation. Induction of ERK5 activation was also observed in response to H_2O2, a well known activator of ERK5, while macrophage activators, such as LPS or Interferon-γ, failed to activate ERK5. Activation of ERK5 following M-CSF administration was peculiar of macrophages as M-CSF failed to activate ERK5 in NIH/3T3 fibroblasts expressing ectopic M-CSF receptor (M-CSF.R). To assess the contribution of pathways downstream of the M-CSF.R, activation of ERK5 was measured in the presence of different pharmacologic inhibitors. Inhibition of PKC isoforms or the serino-phosphatases PP1 and PP2A did not have any effects on ERK5 activation, while several molecules interfering with Src-family kinases activation inhibited M-CSF-dependent ERK5 phosphorylation. Preliminary data performed with siRNA targeting ERK5 seem to indicate that ERK5 is involved in M-CSF-induced macrophage proliferation.

PO-026

RITUXIMAB IN THE TREATMENT OF AUTO-IMMUNE DISEASES

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Background. Standard therapy of autoimmune diseases includes drugs with non-specific immunosoppressive capacities, with several complications such infections and toxicity at the expense of numeorus organs including kidney and liver. Rituximab induces complement- or cell-mediated lymphocytolysis. It blocks proliferation and stimulates apoptosis of auto-reactive lymphocyte clones, underlying auto-immune diseases. We assessed the efficacy of the anti-CD20 monoclonal antibody, Rituximab, as therapy for autoimmune diseases in patients resistant to standard immunosoppressive therapy. Rituximab Treatment Plan: Primary AIHA, Thrombocytopenia, Cryoglobulinema, Pemphigus: Intravenous Rituximab 375mg/m^2 once a week for 4 weeks. Secondary AIHA: Rituximab 375 mg/m^2 every two weeks for two months combined with cyclophosphamide 1 gr ev every two weeks. *Patients, methods and results.* The 23 study participants included: a) 10 cases of autoimmune haemolytic anaemia (AIHA): idiopathic in 4/10 patients(2 AIHA with warm antibodies and 2 with cold and secondary to lymphoproliferative disorders in 6 (3 NHL, 3 CLL). All 10 patients had previously received steroid therapy, and 6/10 had also received cyclophosphamide. Five of the 6 patients with lymphoproliferative disorders (3 CLL, 2 NHL) had received cycles of chemotherapy in the preceding months and 2 had undergone autologous bone marrow transplantation. *Results.* haemoglobin levels increased in all 10 patients and steroids were rapidly suspended. One month after therapy 8/10 patients achieved full response reaching 12 gr/dL haemoglobin. The other 2 achieved two partial response (Hb 10-12gr/dL). Response lasted at least 6 months (median 10 months; range 5-15 months). Rituximab was well tolerated, only 2/10 patients reacted to the first administration with chills and malaise. None asked to suspend treatment. b) 8 cases of auto-immune thrombocytopenia: 6/8 patients had idiopathic thrombocytopenic purpura (ITP), 1 had a lymphoproliferative associated disorder, another had Sjogren's disease. All had platelet (PLT) counts below 10,000/mmc, and had received steroids and high dose, intravenous immunoglobulin. Azathioprine had been administered to 2 in the previous 2 months. Chemotherapy had failed in the patient with lymphoproliferative disorder. Splenectomy had failed in 3 patients with ITP. *Results.* All 8 patients had a good response. 3 months after treatment PLT counts were over 80,000/mmc in 6, with 3 reaching normal levels, and PLT counts were stable at about 40,000/mmc in 2. Remission lasted a minimum of 6 months(median 10; range 6-13). Steroid dosage was reduced in all patients and in 3 patients steroid treatment was suspended after 4 weeks. c) 3 cases of mixed cryoglobulinemia: idiopathic in 1, associated with chronic hepatitis due to HCV infection in 2. All presented cryoglobulinemia-related symptoms e.g. arthralgia, asthenia, petechial lesions of the lower limbs. Previous treatment included interferon-α, plasmapheresis and steroids. *Results.* Rituximab was suspended in 1 patient after 2 doses due to a persistent urticaria-type skin reaction. In this patient cryocrit was reduced from 82.7% to 44.0% but there was little clinical benefit. Cryocrit fell in 1 patient from 156% to under 30% and from 60% to negative in the other. These reductions were associated with disappearance of arterial pain and of cutaneous lesions of the lower limbs. d) 2 cases of Pemphigus: paraneoplastic pemphigus secondary to NHL in 1 with mucosal and cutaneous lesions, and pemphigus vulgaris, with cutaneous lesions, in the other. Previous therapy included steroids and azathioprine (with severe side effects). *Results.* we obtained lesions improvement in both patients with suspension of previous immunosoppressive therapy (steroids in one patient and azatioprine in the other one). Both patients sustained clinical remissione until now (13 months). *Conclusions.* Rituximab appears to be a valuable therapeutic agents in the autoimmune diseases we treated, in patients resistant to standard immunosuppressive drugs.

PO-027

RITUXIMAB FOR THE TREATMENT OF IDIOPATHIC AUTOIMMUNE HEMOLYTIC ANEMIA.

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Idiopathic autoimmune hemolytic anemia (AIHA) is a severe and often life-threatening condition. While steroids represent the first-line therapy option, a variety of other immunosuppressive agents and splenectomy are employed for steroid-refractory cases. The anti-CD20 monoclonal antibody Rituximab has shown to control AIHA in patients with refractory chronic disease. We report our experience on the use of Rituximab treatment in 7 patients with idiopathic AIHA. The patients were diagnosed with idiopathic warm-antibody AIHA at the mean age of 58 years (range 35-81 years). An underlying autoimmune or lymphoproliferative disorder was ruled out in all cases and all patients received steroids (methyl-prednisolone) as first-line treatment. Prior of Rituximab therapy, some patients (no. 1, 3, 4, 6, and 7 in the Table) also received azathioprine, while patients no. 1 and no. 5 received intravenous high-dose immunoglobulins.

Table. Patients characteristics, Hb, lymphocytes (Ly) and platelets (Plts) values pre and post rituximab treatment and follow-up since the initiation of treatment with monoclonal antibody.

UPN	Age/Gender	Hb (g/dL) pre/post	Ly (/uL) pre/post	Plts (/uL) pre/post	Follow-up (days)
1	71/F	5.6/11.4	300/1100	221/226	46
2	35/M	12.8/13.4	2000/2600	170/333	990
3	41/M	8.5/11.5	700/688	198/242	60
4	55/M	7.7/11.5	1800/4950	110/179	150
5	50/M	5.5/12	2500/2000	230/221	30
6	81/F	11/12	900/580	90/123	45
7	62/F	4.3/7.2	1000/950	200/251	90

All patients but two were considered refractory to steroids and/or subsequent immunosuppressive therapy and all were then given weekly Rituximab (375 mg per meter square) for 4 consecutive weeks at a median time of 797 days (range 15-3960 days) since the diagnosis of AIHA. Table shows transfusion-independent Hb (g/dL) levels before and after rituximab treatment. All patients received the scheduled 4 courses. The first infusion side effects were irrelevant. All patients showed an increase in Hb levels in response to rituximab with a mean increment value of 3.3 g/dL (range 0.6-6.5). The hematologic improvement was prompt, appearing by the second or third infusion of rituximab. Three patients required packed red cell transfusions before starting rituximab and all became transfusion-free. At a mean follow-up of 201 days (range 30-990 days) since the treatment of AIHA with rituximab all patients are still alive, 6 (86%) of them in CR and only one (14%) in PR with a persisting moderate hemolysis requiring maintenance treatment with rituximab given as a single dose every 4 weeks (no. 4 in the Table). In conclusion, our results clearly demonstrate that anti-CD20 monoclonal antibody rituximab is an effective and safe alternative treatment option for idiopathic AIHA, in particular for steroid-refractory disease.

PO-028

HEREDITARY HEMOCHROMATOSIS AND SECONDARY IRON OVERLOAD: TREATMENT BY THERAPEUTIC ERYTHROCYTAPHERESIS

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Erythrocytapheresis is effective in removing iron excess in patients with hereditary and secondary hemochromatosis. We have treated 24 patients (aging 39-66 years): 4 affected by C282Y homozygous mutation, 8 H63D heterozygous mutation and chronic hepatitis (5 HBV related and 3 HCV related), 12 patients with chronic hepatitis HCV-related without HFE mutation. Clinical and biochemical data indicated marked iron overload and hepatic failure; all patients underwent liver biopsy that showed cirrhosis and confirmed severe iron overload, moreover the portal hypertension and splenomegaly were echo graphically evident. The C282Y homozygous patients showed the most severe clinical conditions, in fact all of them were also affected by multiorgan failure: diabetes, hypogonadism and hypokinetic cardiomyopathy. The remaining 20 patients showed high serum levels of Ferritin associated with a high Transferrin's saturation, but however lower than the 4 subjects homozygous with hereditary hemochromatosis. All patients were referred to us for counselling since the phlebotomy was contraindicated: in fact some of these patients showed hypotension, causing dizziness and faintness, during phlebotomy or prolonged fatigue after venesection, while other patients cannot undergo to this treatment because of low levels of plasmatic proteins (probably due to liver failure). Erythrocytapheresis was performed using a computer-guided discontinuous flow cell separator in each patient. Our protocol of treatment consists in an therapeutic erythrocytapheresis every ten days until iron depletion; for every apheretic procedure 210±20 mL of packed RBC were removed, while plasma with 250 mL of saline solution were re-infused to the patient. For the patients' safety, procedure parameters were set to achieve a final hematocrit not lower than 34%. The baseline laboratory evaluations was (main ± SD): Transferrin saturation = 77±7%, ferritin = 978±403 ng/mL, alanine transferase = 75±33 IU/L. At the end of treatment all patients achieved iron depletion, showing a Transferrin saturation lower than 40%, a ferritin lower than 100 ng/mL, and a normalization of alanine transferase serum levels. Erythrocytapheresis is effective in removing iron excess such as phlebotomy, but, in our opinion, it presents three advantages: 1) hypovolemia is absent because the collected blood volume is compensated by autologous plasma and saline solution; 2) the frequency of apheretic procedures is decreased respect to (weekly) phlebotomy, because for every erythrocytapheresis the amount of red blood cell removed is higher than phlebotomy; 3) in these cirrhotic patients, erythrocytapheresis permits the saving of platelets, plasma proteins and clotting factors. Erythrocytapheresis, compared to phlebotomy, presents two disadvantage: 1)it requires adequate equipment and trained staff; 2) it is more expensive. In conclusion, for these last reasons, we suggest that this procedure should be proposed for selected patients whose clinical conditions do not permit the execution of the phlebotomy.

PO-029

PLATELET TRANSFUSION AND RISKS OF BACTERIAL CONTAMINATION

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Bacterial contamination of hemocomponents represents a serious risk for patients. In fact about 10% of transfusion-associated deaths are caused by sepsis due to bacterial contamination of cellular blood products. In particular, (both apheresis- and whole blood- derived) platelet concentrates are the most frequently implicated hemocomponents in this kind of transfusion complications. The reason of this observation is that platelets are stored in conditions allowing the bacterial growth: at room temperature (22±2°C), in a large volume of plasma (50-70 mL) and in a particular bag that allows oxygen exchange. The two main sources of bacterial contamination are the Gram-positive skin flora, introduced into the blood unit during collection, and an occult (asymptomatic and unknown) donor bacteremia. A review of literature data suggests that, by culturing of platelet concentrates, about 70-80% of isolated bacteria are Gram-positive (mainly Staphylococcus and Streptococcus), while Gram-negative organisms comprise the remaining rate (prevalently Serratia marcescens, Salmonella and Yersinia enterolitica). On the contrary, Gram-negative bacteria are involved in 40-80% of deaths due to transfusion-related sepsis. At the time of blood donation, the level of contamination is relatively low, but during the storage the bacteria can proliferate to levels of 10 million/mL or greater. The transfusion of a contaminated unit can cause bacteremia, fever, chills, hypotension, nausea, vomiting,

diarrhea, and oliguria, which may progress to sepsis and ultimately multi-system organ failure and death. However, in the majority of the cases, a contaminated transfusion may produce no clinical consequences. The severity of the reaction mainly depends on the species and the concentration of involved micro organism and on the status of the immune system of the patient. Considering the clinical significance and the fatal risk of bacterial contamination for patients, two precautionary measures must be taken: 1) to avoid the not necessary transfusions, especially of random platelet concentrates; 2) to reduce the amount of bacterial contamination. The first point mainly involves hematologists, pediatrics and oncologists that largely prescribe platelet transfusions. The second point is linked to the activity of blood donation: a stringent donor screening; disinfection of the skin (at the phlebotomy site) prior to donation; diversion of the first amount of the collected blood into an accessory bag (used for laboratory testing) in order to remove the skin plug, produced by the needle as it passes through the skin; avoiding the not necessary laboratory manipulations and the use of a sterile connector are all measures routinely used in Transfusion Centres to reduce the bacterial contamination. Nevertheless the contamination of blood products continues to be observed and fatalities due to septic reactions have been reported. Nowadays, on the basis of international reports, it has been theoretically estimated that 1:2.000-3.000 blood units are contaminated with bacteria, while transfusion related sepsis is less common (1:20.000-85.000 blood units), at the end an approximate incidence of 1 fatality on 500.000 transfusions has been postulated. In consideration of about 134.000 blood donation carried out in Campania in 2005, we can calculating that, in the last year, about 45-67 contaminated blood units have been collected in our region and 2-7 transfusion related sepsis have been taken place. As a similar residual risk of sepsis and fatality is largely greater than the combined risk of transfusing HIV, HBV and HCV, it may be judged unacceptable and an improving of blood safety needs. New strategies, to reduce this complication and improve transfusion safety, are to detect (by rapid, sensible, specific and inexpensive pre-storage and/or pre-transfusion screening tests) or to limit (by pathogen reduction-inactivation, with methylene blue, riboflavin, solvent-detergent treatment and/or the use of psoralens and ultraviolet light) the bacterial contamination in all platelet concentrates. Implementation of these methods may allow the extension of platelet storage from 5 to 7 days, thus also improving the availability of this still (unfortunately) limited resource.

PO-030

ADVANTAGES IN THE USE OF RED BLOOD CELL CONCENTRATES FROM MULTICOMPONENT COLLECTION (MCC) IN NEONATAL TRANSFUSIONS

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The MCC permits to obtain hemocomponents with highly standardized quantitative and qualitative characteristics. Erythrocyte transfusion is widely used in neonatal intensive care units for acute or chronic pathological conditions. Aim of this study was to evaluate the effectiveness of transfusion in newborns using paediatric erythrocyte concentrates obtained by MCC or by processing of whole blood. In our neonatal intensive care units, 252 newborns (with a gestation age of 23-37 weeks and a weight from 580 to 3.340 g) were transfused. Clinical indications were shock, sepsis and/or anaemia with the following criteria: A) hematocrit (Hct) <20% or hemoglobin (Hb) <7g/dL and reticulocytes <4%; B) Hct <30% or Hb <10 g/dL in these conditions: O2 required <35%, recurrent apnoea and bradicardia, cardiac rate >180 bpm and respiratory rate >80 bpm for more 24 hours; C) Hct <35% or Hb <12 g/dL with severe respiratory distress. The RBC's transfused volume was calculated using the following formula: weight (Kg) x blood volume (100 mL/Kg if premature or 80 mL/Kg if at term newborn) x (desired Hct – observed Hct)/Hct of transfused unit. In order to avoid a circulatory overload, the RBC concentrates were packed with high Hct (≥ 80%). For the same reason, the rate of infusion was 3-10 mL/Kg/hr. The therapeutic objective was to achieve an Hct of 50%. Hct, sodium (Na), potassium (K) and pH were measured before and after the transfusion and after 24 hours, 72 hours and 1 week too. 11 newborns were transfused with 34 RBC-pheresis (MCC group): 3 were only transfused one time, 1 patient received 9 transfusions, while remaining 7 children received a mean of 3,14±0,38 blood units. These patients have been compared with 241 newborns transfused with 321 paediatric units obtained by processing of whole blood (WB group): 148 patients received 1 transfusion, while 93 received from 2 to 11 blood units. No significant statistical change, in both groups, occurred in mean pH, Na and K, probably due to small transfused blood volume. At the end of transfusion and after 24 hours the mean Hct resulted increased in all newborns, achieving values higher than 50%. After 72 hours the mean Hct still resulted significantly increased in both groups, but reduced in respect of previous values. In the last control, after 1 week, the mean Hct were furtherly decreased, but mean Hct in MCC group was significantly higher than in WB group ($p<0.05$). On the basis of our data, we can affirm that the attended post-transfusion increase of Hct is obtained using packed RBC units produced either from whole blood or by MCC. Both products represent an efficient therapy in the treatment of acute severe anaemic conditions at birth with no significant statistical differences among them immediately after transfusion. Nevertheless, during the long term post-transfusion follow-up of newborns, a therapeutic advantage of MCC may be exist (probably) diminishing the blood request and (probably) avoiding the exposure of the newborn to multiple donors.

Lymphoproliferative Disorders

PO-031

THE OCCURRENCE OF A PRIMARY CUTANEOUS DIFFUSE LARGE B-CELL LYMPHOMA IN A PATIENT WITH CHRONIC MYELOID LEUKAEMIA TREATED WITH IMATINIB MESYLATE

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The major pathogenetic event of chronic myeloid leukaemia (CML) is the constitution of the Philadelphia chromosome (Ph) and the BCR-ABL oncogene. Imatinib mesylate is a potent, specific inhibitor of BCR-ABL tyrosine kinase and can induce hematologic, cytogenetic and molecular response. Non-Hodgkin lymphoma (NHL) occurring as a synchronous malignancy with CML is rare; a review of the literature identified two different subtypes of malignant lymphomas arising in patients with an antecedent or concurrent diagnosis of CML: the most common are T cell lymphomas with an immature thymic phenotype, while peripheral B cell lymphomas are more rare. We describe the occurrence of a primary cutaneous diffuse large B cell lymphoma in an old man with CML treated with imatinib mesylate. A 78-year-old patient was diagnosed with chronic phase CML. Bone marrow conventional cytogenetic showed t(9;22) in all metaphases with ad additonal abnormality: the lack of the Y cromosome. Fluorescent in situ hybridization (FISH) confirmed the disease-causing ABL to BCR translocation. According to Sokal score the patient was an intermediate risk (0.97). He started imatinib mesylate (400 mg/die): a complete hematologic response was obtained within one month. Because of a maculo-papular cutaneous rash of the limbs starting about 5 weeks after the beginning of treatment (grade II according to NCI/NIH common toxicity criteria), there was a temporary dose reduction with resolution of skin lesions. The 3-month bone marrow evaluation showed a major cytogenetic response: –Y persisted in some metaphases in which Ph chromosome was not present; interphase FISH showed BCR/ABL fusion gene signal in 1% of 400 nuclei. About 8 months after the diagnosis of CML we observed an enlargement of the left breast, in the nipple region, that was initially interpreted as gynaecomastia, that is a possible side effect of imatinib therapy. Then it evolved into a nodular and vegetant necrotic cutaneous neoformation with irregular acies (Figure 1).

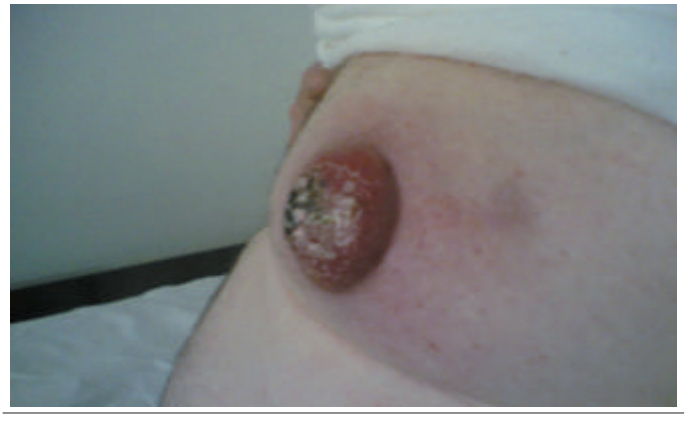

Figure 1.

There was a plaque-like infiltration of the surrounding skin; echographic examination showed a diffuse abscessual infiltration. Histopathology and immunohistochemistry was consistent with NHL; because of negative staging procedures the diagnosis was primary cutaneous diffuse large B-cell lymphoma, leg type. The patient has recently started chemotherapy accordind to CHOP regimen plus Rituximab. The development of CML and NHL in this patient suggests probably a genetic predisposition, although other factors could have had a contributory or synergistic role, including environmental exposures and the therapy with imatinib mesylate (even if at the time of lymphoma diagnosis, the patient had only 9 months of therapy). The loss of the Y chromosome is a feature of haematologically normal bone marrow in elderly males, but it can be found also in haematological malignancy. Prognostic association with survival of -Y at diagnosis of disease appears to be neutral or favourable; it has a worse significance when occurring during the course of the disease. NHL occurring in CML may represent an unrelated neoplasm. Accurate discrimination (for example with FISH) between an extramedullary localized CML blast phase and a genetically distinct NHL is essential for determining subsequent therapy.

PO-032

RITUXIMAB INDUCES EFFECTIVE CLEARANCE OF MINIMAL RESIDUAL DISEASE (MRD) IN MOLECULAR RELAPSES OF MANTLE CELL LYMPHOMA

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Mantle Cell Lymphoma (MCL) is one of the worst subtypes of B-cell lymphoma. In MCL molecular remission (MR) is associated with improved outcome (Pott et al Blood 2006). If MR is lost patients are at high-risk of relapse. Rituximab has been employed in MCL and it seems to be beneficial in combination either with conventional chemotherapy or with autologous stem cell transplantation (ASCT). With the latter treatment MRs were also frequently reported, as opposed to patients treated with Rituximab-free autografting regimens. However, the value of this drug is not exhaustively established. In this study we describe the molecular and clinical follow-up of four patients with post-allograft molecular relapse (M-rel) treated with Rituximab. *Methods.* M-rel was defined as PCR-positivity in two consecutive bone marrow samples of a patient with previous MR and ongoing clinical complete remission (CR). In our patients M-rels occurred at 3, 6, 39 and 52 months, respectively. M-rels were confirmed by direct sequencing of the t(11;14) for two patients and IgH rearrangement for the other two. Minimal residual disease (MRD) was monitored by qualitative and Real-time quantitative PCR. All patients received four Rituximab courses at the dosage of 375 mg/sqm, with two additional infusions if MR was not reinduced. Results. All patients were in CR throughout the whole observation period, that is respectively 18, 55, 71 and 72 months from transplantation. All the M-rels were confirmed to be the same original tumor clone seen at diagnosis. Rituximab treatment was effective on all the four M-rels as all patients reverted to a PCR-negativity following four or six Rituximab courses. This status is currently maintained in three patients at 3, 6 and 18 months. Only one patient experienced a second molecular relapse 24 months later, that was again sensitive to Rituximab. Figure 1A and 1B shows the molecular follow-up of our patients by qualitative and quantitative PCR.

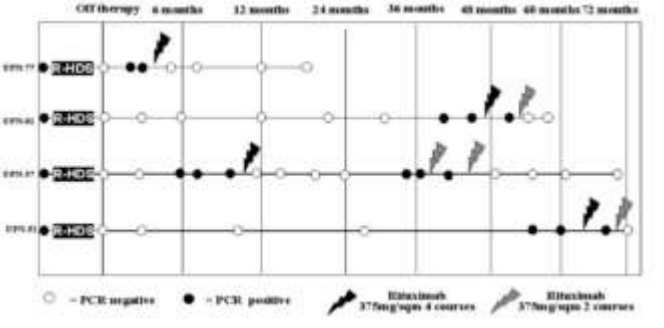

Figure 1A.

Interestingly, Real-time PCR showed stable or increasing tumor burden before Rituximab delivery, followed by progressive clearance of tumor cells after treatment. This reduction resulted in a re-achievement of MR after four courses in two patients and after six courses in the other two. Discussion.

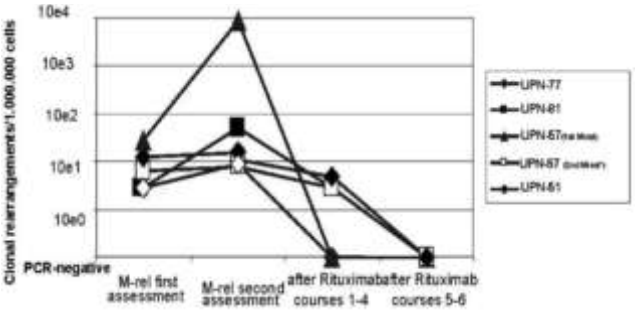

Figure 1B.

Our study shows, for the first time, that Rituximab can reinduce MR in MCL patients experiencing M-rel following intensified Rituximab-containing autografting regimen. This suggests that residual tumor cells are still sensitive to this agent. None of the patients, re-entering MR, experienced clinical relapse. Our results indicate that Rituximab is active against residual MCL cells and suggest that molecularly tailored maintenance therapy needs to be investigated in clinical trials.

PO-033

POLYMORPHISM IN CYTOKINE GENE PROMOTERS AND RISK OF HODGKIN'S LYMPHOMA

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Introduction. Hodgkin's lymphoma (HL) is a primary solid malignancy of the immune system characterized by an abundant component of inflammatory cells and only by a limited number of the putative neoplastic subpopulation of Hodgkin and Reed-Sternberg (HRS) cells. This predominant immune infiltrate in HL tissues is known to be associated with an exuberant production of cytokines and chemokines Single nucleotide polymorphisms (SNPs) have been identified in the 5'-promoter region of cytokine genes and they may be the reason for inter-individual differences in cytokine expression. The aim of our study was to assess whether these genetic polymorphisms might influence HL risk. *Methods.* To test our hypothesis we performed a case-control study of 204 LH patients (median age 32 years, range 14-77 years; 90 females and 114 males) and 204 healthy individuals matched for age and sex, genotyping polymorphisms in the IL-10 gene (T-3575A, G-2849A, C-2763A, A-1082G and C-592A), TNF α (C-863A and G-308A) and IL-6 (G-176C) genes. Polymorphisms were analyzed by nested or by mismatched PCR-RFLP. A multiplex PCR-RFLP assay was developed for simultaneous genotyping of IL-10/-1082, IL-10/-592 and TNF/-308 polymorphisms. We also assessed the IL-10 serum levels in a subgroup of 58 HL patients and in 65 normal controls using a standard ELISA technique (DuoSet Human IL-10, R&D Systems). *Results.* Allele frequencies and genotype distribution in the control group were as expected for normal individuals. Female HL patients had a significant higher frequency of the IL-10 /-1082 A allele in comparison to controls (71% vs 58%). This resulted in an odds ratio (OR) of 1.32 (95% C.I. 1.04-1.68; p=0.021) for carriers of the A allele to develop HL. In addition, carriers of the -1082A/-592A haplotype were at increased risk for HL (OR: 1.36, 95% C.I. 1.05-1.76; p = 0.027). Reconstructing haplotypes in the IL10 promoter region using the PHASE program, we observed a limited haplotype diversity in both controls and patients, with 5 common haplotypes (TGCAA, TGCAC, AGAGC, TGCGC, AAAGC) describing 83% and 81% of the observed variations, respectively. Carriers of TGAAA and TAAGC haplotypes were at lower risk for HL (OR: 0.10, 95% C.I. 0.00-0.69, p=0.009 and OR: 0.15, 95% C.I. 0.02-0.67; p=0.01, respectively). By contrast, the TAAAA haplotype increased the risk for HL (OR: 7.76, 95% C.I. 0.91-360.32, p=0.047). IL-10 serum levels were significantly higher in patients compared to controls (96+22 pg/mL vs. 46±10 pg/mL, p=0.028). Considering the 95% upper C.I. of the controls as cut-off, IL-10 levels were found to be elevated in 43% of patients. We found that female homozygous carriers of the allele -1082A of the IL-10 gene had significant higher levels of IL-10 than female carriers of at least a G allele (61±16 pg/mL vs 17±5 pg/mL, p=0.005). *Conclusions.* our study suggests that the IL-10 genotype may be a risk factor for Hodgkin's lymphoma in at least a subset of patients. These studies point to the importance of the genetic background of the host in the biology of the disease.

PO-034

FROM HODGKIN LYMPHOMA TO SECONDARY ACUTE LEUKEMIA: EVALUATION OF GENOMIC PATTERN

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Hodgkin's lymphoma (HL) is a heterogeneous lymphoid malignancy curable in more than 80% of cases. Its morbility and mortality are mostly contingent upon chemo- and radio-therapy side effects including cardiovascular or pulmonary diseases, infections and second malignancies. Indeed, the high incidence of secondary cancers and leukemias in potentially cured LH patients supports that genomic instability and defective DNA repair system have a crucial role in secondary cancerogenesis/leukemogenesis processes as they let the emergence of genomic aberrations and clonal cell evolution towards a transformed phenotype. The aim of our study was to define the genomic profile associated with HL evolution towards secondary leukemia. To the purpose, formalin-fixed/paraffin-embedded biopsies from 4 LH patients who developed a secondary acute myeloid leukemia (AML) were analyzed by mean of array-based comparative genomic hybridization (aCGH). This method allows the identification of changes in DNA sequence copy number (amplifications or deletions) at high resolution. The microarrays used in our study contain 287 genomic targets involved in most human cancers, including oncogenes, tumor-suppressor genes and DNA sequences localized within chromosomal regions most frequently rearranged. DNAs from lymphonode biopsies of LH patient who developed AML were compared with pooled DNAs from reactive and LH lymphonodes (comparable for histotype and follow-up) from cured patients. CGH profiles of single LH patients were extensively altered. However, they shared a common pattern of structural genomic alterations. Bioinformatic analysis of CGH results performed with dedicated softwares let distinguish statistatically significant (ratio >1,2) amplifications relative to 3 DNA sequences (AFM137XA11, FGFR1, PPABP) and statistatically significant (ratio< 0,83) deletions in 4 (AFM217YD10, FGR(SRC2), GATA3, TOP1, WT1). CGH data relative to the above mentioned sequences were further validated by FISH and immunohistochemistry. In spite of the low number of LH patients our results suggest that the evolution to AML in LH patients may be genetically determined. The role of single genes in leukemic transformation requires further investigation.

PO-035

COMPARATIVE GENOME-WIDE PROFILING OF POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS AND DIFFUSE LARGE B-CELL LYMPHOMAS

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Post-transplant lymphoproliferative disorders (PTLD) are a major complication of solid organ transplantation, representing a cause of severe morbidity and mortality among transplanted patients. PTLD are generally of B-cell origin and comprise a histologic spectrum ranging from polyclonal hyperplasia to overt lymphoma. Based on WHO classification, PTLD are classified into early reactive lesions, polymorphic PTLD (P-PTLD) and monomorphic PTLD, comprising diffuse large B-cell lymphoma (PT-DLBCL) and Burkitt/Burkitt-like lymphoma (PT-BL/BLL). Apart from Epstein–Barr virus infection, knowledge of the pathogenesis of monoclonal PTLD is limited. The clinical variability of the disease, the frequent extranodal involvement and the wide spectrum of histological features make the diagnosis of PTLD difficult by conventional strate-

gies. On these grounds, insights into the biology and the molecular features of PTLD are urgently needed. Powerful genome analysis techniques, such as genome-wide DNA profiling (array comparative genomic hybridization), based on microarray DNA technology can improve our understanding of PTLD pathogenesis. Whole genome profiling was performed on 20 monoclonal PTLD (4 P-PTLD, 13 PT-DLBCL and 3 PT-BL) and 25 DLBCL from immunocompetent patients (IC-DLBCL), using the Affymetrix GeneChip Human Mapping 10 k 2.0, an oligonucleotide microarray targeting about 10.000 single nucleotide polymorphisms. Recurrent lesions were detected in all samples. Chromosomes 5p, 9p and 11p were commonly gained in PT–DLBCL, whereas, in order of frequency, chromosome 18q, 7q, 3q, and 12 were the most common gains in IC-DLBCL. Chromosome 12p was the most frequent target of deletion among PT–DLBCL, followed by 4p, 4q, 12q, 17p and 18q. IC-DLBCL had frequent losses of 6q, 17p, 1p and 9p. The Loss of Heterozygosity (LOH) pattern was characterized by the involvement of chromosome arms 10q, 1q, 9p and 11q among PT-DLBCL and 13q and 17p in IC-DLBCL. In PTLD, LOH did not always match DNA loss. In particular, chromosome 10 seemed to be targeted by uniparental disomy. Small alterations, involving both known (BCL2 and PAX5) and unknown (11q25 and 6q25.3) genes were identified. Loss of 11q25 region was found in all 3 PT-BL/BLL, in 2 IC-DLBCL and in 3 PT-DLBCL. The gain of 6q25.3 region was detected in all 3 PT-BL/BLL and in 2 IC-DLBCL. The 11q25 region contains the neurotrimin (HNT) gene, a member of the IgLON family of Ig domain-containing glycosylphosphatidylinositol (GPI)-anchored cell adhesion molecules. The 6q25.3 region contains only the ZDHHC14 (zinc finger, DHHC-type containing 14) gene. ZDHHC14 is expressed by normal B cells and by different DLBCL cell lines. It contains the DHHC/NEW1 domain, supposed to be involved in protein-protein or protein-DNA interactions and palmitoyltransferase activity. In conclusion, these data suggest that PTLD share common genetic aberrations with IC-DLBCL. The demonstration of 9p13 amplification emphasizes the importance of PAX5 in PTLD pathogenesis. The combination of DNA copy number and LOH assessment leads to the hypothesis that uniparental disomy may be a potential mechanism in B-cell lymphomagenesis. Finally, the ZDHHC14 gene appears to be a new putative cancer gene in lymphomas, possibly targeted by copy number changes due to polymorphisms or somatic events.

PO-036

CIRCULATING Vδ1 T LYMPHOCYTES PRODUCING IL-4 ARE INCREASED IN LOW GRADE NON HODGKIN LYMPHOMAS EXPRESSING UL-16 BINDING PROTEINS

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Low-grade non-Hodgkin lymphomas (NHL), and chronic B cell lymphocytic leukemia (B-CLL) with lymph node involvement, frequently relapse or become resistant to chemotherapy. Several studies have demonstrated a role for human γδ T lymphocytes in the recognition of transformed cells. Among these lymphocytes two main subsets are known: the circulating Vδ2 subset is capable of killing myeloma and Burkitt lymphoma cells, and the Vδ1 subset, mainly located in the mucosal associated lymphoid tissue and implied in the defence against epithelial cancers. We recently described that Vδ1 T cells are increased, and display anti-tumor cytolytic activity, in low risk B-CLL patients whose B lymphocytes expressed the UL-16-binding protein ULBP3. In this study, we analyzed 23 patients with low grade NHL, 4 mantle (MT), 4 marginal zone (MZ), 15 follicular (FL), compared to 10 high risk (HR) B-CLL with lymph node involvement and to 4 diffuse large cell lymphomas (DLCL). A significant increase in circulating Vδ1 T lymphocytes producing interleukin-4 (IL-4) was found in most FL, MT and MZ NHL patients, at variance with DLCL and HR B-CLL. IL-4 was also detectable in the sera of the same patients. In the majority of NHL (20/23) with increased circulating Vδ1 T lymphocytes, B cells expressing ULBP2 or ULBP3 or both were found in peripheral blood, bone marrow and/or lymph nodes. Of note, in HR B-CLL or in DLCL, where leukemic cells were ULBPs negative, no γδ T cell increase was found. Moreover, Vδ1 T lymphocytes from FL NHL patients proliferate in response to ULBP2+ and ULBP3+ lymphoma cells. Finally, patients with high expression of ULBPs, increased circulating γδ T lymphocytes and high levels of serum IL-4 showed stable disease in a one year follow up, at variance with patients with low circulating γδ T cells and undetectable expression of ULBPs. Our results point to a role for Vδ1 T lymphocyte in the anti-tumor defence against low grade lymphomas and highlight a potential therapeutic use for this T cell subset. The use of γδ T cells in immunotherapy has been proposed in humans; their function might be potentiated by the use of drugs able to induce or upregulate the molecules recognized by either Vδ1 or Vδ2 T cell subsets, such as transretinoic acid (ATRA) or biphosphonates. Indeed, we have previously reported that ATRA upregulates the expression of ULBP3 on B-CLL; accordingly, preliminary experiments on two DLCL showed that ATRA can induce the expression of ULBP3 on lymphoma B cells as well. Interestingly, it has been reported that ATRA has a negative effect on B cell growth in NHL, thus further supporting the use of this drug in NHL therapy.

PO-037

A CASE OF FAMILIAL HODGKIN LYMPHOMA IN HUMAN LEUKOCITE ANTIGEN CLASS I IDENTICAL SIBLINGS

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Hodgkin disease is a well defined hemopoietic malignancy but little is known about the aetiology; probably involves both genetic factors and undefined external agents. Some studies estimate that approximately 4,5% of HD cases occur as familial diseases, nevertheless available evidence points to a viral infection role in the development of HD; particularly EBV genome have been detectable in about 35% of HD cases. We report a familial HD case in siblings with identical HLA class I. The first sibling, the younger sister, began in 1998 with a diagnosis of classical HD nodular sclerosis, stadium III B. She was treated with four series of ABVD followed by involved field RT, resulting in complete remission. Two years later the patient relapsed within mediastinic lymphadenomegalies and was treated with allo-transplant procedure from HLA identical brother. The patient is presently in complete remission. In 2006 the older brother, her staminal cells donor, presented an enlarged cervical lymph node; histopathology showed classical HD nodular sclerosis stadium IA. He is actually under treatment. This is a case of rare but well documented familial clustering of HD, it suggest that HLA genes might play an important role in HD aetiology, also in other studies a significant increase of HD in certain HLA was found; it may be associated with an impaired immune response of these identical haplotypes among affected first-degree relatives to environmental agents.

PO-038

EFFICACY OF RITUXIMAB PLUS HYPER-CVAD REGIMEN IN MANTLE CELL LYMPHOMA IS INDEPENDENT ON FCGRIIIA AND FCGRIIA POLYMORPHISMS.

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Mantle cell lymphoma accounts for 3-10% of all non-Hodgkin's lymphomas, with median OS not exceeding 3-4 years. Rituximab in combination with Hyper-CVAD appeared the most promising regimen; thus, we adopted it as first-line treatment strategy in a series of 24 patients. In addition to the evaluation of clinical success of the regimen, we investigated a possible role of polymorphism in IgG Fc receptor, FCγRIIIa and FCγRIIa. The frequencies for FcγRIIIa-158 were as follows: V/V=4/24 (17%); V/F=16/24 (66%); F/F=4/24 (17%).Those for the FcγRIIa-131 polymorphism: H/H=11/24 (46%), H/R=9/24 (37%), R/R=4/24 (17%). The overall response rate after the first two Hyper-CVAD cycles was 87.5%, with 44% of CRs. The quality of response was not influenced by the assessed polymorphisms. Two-year PFS was 78% for patients -158V/V vs 75% for cases carrying phenylalanine (p=0.88). When the FcγRIIa polymorphism was assessed, the 2-year PFS was 82% for

patients -131H/H vs 75% for cases carrying arginine (p=0.26). Eighty-three percent of cases achieved the PCR-negativity: the progression rate was significantly influenced by the minimal residual disease clearance, with 12% of progression rate in the subgroup of PCR-negative cases versus 67% of cases PCR-positive (p=0.008). Nevertheless, the achievement of the PCR-negativity was not significantly influenced by Fcγ polymorphisms. Results confirm that Rituximab plus Hyper-CVAD is an effective regimen for the induction of prolonged remission in patients with aggressive MCL. The observation that neither response rate and long-term outcomes nor minimal residual clearance are influenced by the Fcγ polymorphisms supports the use of Rituximab in all phases of treatment.

PO-039

PURGING *IN VIVO* WITH MONOCLONAL ANTIBODIES AND AUTOLOGOUS STEM CELL TRANSPLANTATION IN POOR PROGNOSIS PATIENT WITH LYMPHOPROLIFERATIVE DISEASE

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Autologous stem cell transplantation is a efficacy therapy for limphoproliferative disease. However a concern with the procedure is the potential of malignant cells to reinfuse with stem-cell graft. In the past five year, investigators have used rituximab to purge malignant cells *in vivo* without any manipulation *in vitro*. From April 2003 to May 2006 we have treated with Autologous stem cell transplantation, purged *in vivo* with monoclonal antibodies, 13 patients (2 F; 11 M median age: 56 years) with limphoproliferative diseases to poor prognosis (2 Burkitt lymphoma; 3 mantle cells; 3 CLL; 1 NHL- peripheral T cells; 2 follicular and 2 large cells) and we have evaluated the results and the feasibility. In all patients, the purged *in vivo*, has been effected administering a dose of monoclonal antibodies (anti CD20 in B-NHL and anti CD52 in CLL and T-NHL) before the harvest and after the infusion of the stem-cells. To the transplantation 3 patients were in CR (2 Burkitt lymphoma and 1 mantle cells) 7 in PR (1 CLL; 2 mantle cells; 2 follicular and 2 large cells lymphoma) and 3 in resistant disease (2 CLL and 1 NHL peripheral T cells) All patients have harvest (median CD34:4 $\times 10^6$/Kg) and median minimal residual disease in the harvest has been <to 2%. All the patients have been conditioned with BEAM and the graft are documented in 12/13 patients (1 patient is dead to the day +4 for gastric haemorrhage) with neutrophils> 1000 in media to day + 14 (range 10-19 days). After transplantation 12/13 patients were in CR, a day +60 the MMR in bone marrow was <0, 5% (range 0-0, 3%). With a median follow-up of 8 months after transplantation (range 2-36) 11/12 patients are in CR (one patient with burkitt lymphoma is relapsed extra-nodular at months +3 and died for disease a months + 5 after transplantation). One patient (CLL) is died at months + 7 for interstitial pneumonia. The DFS and EFS projected at 36 months are of the 85% and 75% respectively. In conclusion the purging in alive with antibodies monoclonal, effected during the harvest that immediately after the infusion of the stem-cells, allows to get besides a graft with least residual disease in this cohort (patients with poor prognosis) and the preliminary results they seem excellent. The principal problem in these patients have been primarily the infectious and gastrointestinal complications, these has been correlated to patients much treated and in disease. These data suggest treating in first line, with transplantation of stem-cells purged *in vivo* with monoclonal antibodies to eradicate the MRD, patients to poor prognosis or with chronic limphoproliferative disease.

PO-040

THERAPY WITH BORTEZOMIB IN PATIENTS WITH REFRACTORY HODGKIN AND NON-HODGKIN LYMPHOMA

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Pharmacological inhibitor of the proteasome shows antitumor activity both *in vitro* and *in vivo*. The proteasome, a multicatalytic proteinase complex, is responsible for the majority of intracellular protein degradation. The Bortezomib has significant efficacy against Multiple Myeloma and promising activity in B-cell non Hodgkin Lymphoma. We reported the experience of the therapeutic use of Bortezomib in four patients, two affected by Mantle Cell Lymphoma (MCL) and two by Hodgkin Lymphoma. Mantle cell lymphoma is a mature B-cell lymphoma with an aggressive course and conventional chemotherapy has little efficacy. The patients with Hodgkin lymphoma (histological type : lymphocitic predominance and nodular sclerosis, Clinical Stage II B) initially received six cycles of ABVD resulting in a partial remission. Subsequently the autologous stem cells transplant was performed, but we didn't observed any response. Therefore the patients were treated with other two lines of polychemotherapy with no significative improvement. A patient had a five years history of lymphoma and the other one three years history of the same disease, when were treated with Bortezomib at 1.3.mg /m^2 on days 1,4,8,11 for two cicles. They didn't show any response to the therapy. The patients suffering from MCL were : a 45-years old female, whose disease was diagnosed on march 2003 (CS IIB) and a 75-years old female, the diagnosis made in september 2003 (CS IIIB with extranodal localitation). The first patient had originally received six cycles of Rituximab plus CEOP obtaining a partial remission. Then four cycles of R-FND were administered with progression of the disease because of a bone marrow involvement. Subsequently she received R-IEV for five cycles with stable disease. The patient was then treated with Bortezomib at 1.3.mg/m^2 on days 1,4,8,11 for six cycles, resulting in a rapid clinical response, on restaging minimal disease was appreciable only by PET scintigraphy. Consolidation therapy with autologous stem cells transplantation was performed and restaging at three months didn't show evidence of disease (PET- negative). After nine months the patient appeared in complete remission. The second patient had initially received three cycles of FLU-CY resulting in no response. Subsequent treatment with four cycles of CNOP plus radiotherapy on the extranodal lesion produced a partial response. The patient began a treatment with Bortezomib at 1.3 mg/m^2 on days 1,4,8,11 for four cycles, the same interrupted for gastrointestinal toxicities grade III. The valutation of the response showed a reduction of adenophaty > 50%. In our experience, Bortezomib has shown no efficacy in Hodgkin Lymphoma primary refractory, but we have observed a partial and a complete remissions in patients suffering from MCL who were refractory to previous therapies. Recent studies have shown *in vitro* sensitivity to Bortezomib in 4 MCL cell lines. These findings and preliminar clinical experiences induces to further studies to evaluate the clinical impact of the Bortezomib alone or in association with cytotoxic or biological agents, in the treatment of MCL.

PO-041

DIFFUSE LARGE B-CELL-NON HODGKIN LYMPHOMA ORIGINATING IN AN INTRAMAMMARY LYMPH NODE: A CASE REPORT

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Non-Hodgkin's lymphoma (NHL) of the breast may be primary or secondary. Both are rare and there are no morphological criteria to establish the differential diagnosis. Only three cases have been previously published. We report a case of a 76-year-old woman who presented with an intramammary lymph node, occasionally found on mammography due to a screening test (2005 April). Mammography showed an enlarged intramammary lymph node, diameter 1,2 centimeters, in the upper outer quadrant of the right breast. The left breast was normal. A biopsy from the enlarged intramammary lymph node was taken. Histological examination revealed a diffuse large B-cell NHL originating in a intramammary lymph node. No peripheral lymphadenopathy or hepatosplenomegaly was found. Hematological examination and bone marrow were normal. We proposed to the patient a protocol of chemotherapy with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) for three courses, to be followed by right breast and ipsilateral axilla radiation; after the second course of therapy (2005 December) the patient was admitted to the hospital for bacterial infection of the right leg which resolved by broad spectrum antibiotic therapy. After dismission from the hospital, the patient refused to complete the protocol of chemotherapy and radiation therapy (2006 January). Since 2006 April, has been no evidence of recurrence on nodal or extranodal disease, both clinically and radiologically. *Conclusion.* We report the fourth case of primary NHL originating in an intramammary lymph node. The involvement of intramammary lymph node by lymphoma is rare, it should be included in the differential diagnosis when an enlarged intramammary lymph node is found.

Acute Lymphoblastic Leukemia

PO-042

CASE REPORT OF CO-EXISTENCE OF DOUBLE FUSION TRANSCRIPTS (BCR-ABL, E2A-PBX1) IN T-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA (ALL)

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Acute lymphoid leukaemia (ALL) is associated with many chromosomal abnormalities leading to formation of fusion transcripts, which act as oncogenes. Each oncogenes is believed to be a different molecular entity and they lead to Leukaemogenesis by different pathways. Although fusion oncogenes have been reported to be co-existed with other cytogenetic abnormaties, more than one fusiuon transcripts have never been reported in the same patient at the same time. Here we report co-existence of BCR-ABL[t(9;22)(q34;q11)] and E2A-PBX1 [(1;19)(q23;p13)] fusion genes at the same time in a paediatric ALL patient showing T-cell ALL. Both fusion genes were detected by a very sensitive RT-PCR assays and confirmed by other molecular cytogenetic techniques. T-cell ALL has also been rarely associated with BCR-ABL and E2A-PBX1 fusion genes. As both BCCR-ABL and E2A-PBX1 fusion genes lead to leukaemogenesis by different pathways, such patients may be very diffucult to treat even with targetted protein tyrosine kinase inhibitors like Imatinib. Further exploration of this patient can provide very valuable information which will help not only understanding biology of leukaemogenesis but also to discovery of effective therapeutic agents.

PO-043

E2A-PBX1 SILENCING REDUCES EB-1 AND WNT16B EXPRESSION IN T(1;19)+ LEUKEMIA CELLS

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Background and Objective. One of the most common non-random translocation in childhood pre-B acute lymphoblastic leukemia (ALL) is t(1;19)(q23;p13), usually resulting in E2A-PBX1 gene expression. Since the role of this chimeric gene during leukemogenesis is not yet fully understood, an approach to investigate its function is to selectively deplete the E2A-PBX1 protein in pre-B leukemic cells and to study the consequences of E2A-PBX1 inhibition. *Design and Methods.* The efficacy of anti-E2A-PBX1 siRNAs has been tested in this study in 697 and RCH-ACV pre-B leukemia cell lines. Transfection was monitored by fluorescence microscopy and FACS. The specific downregulation E2A-PBX1 mRNA expression was measured using real-time quantitative PCR, while the reduction of the correspondent fusion protein level was assessed by Western Blot analysis. To better understand the role of E2A-PBX1 in human pre-B cells leukemogenesis the study focused also on genes whose expression invariantly accompanies the t(1;19) translocation, and their transcripts were detected by SYBR Green PCR. *Results.* The downregulation induced by anti-E2A-PBX1 siRNA in the fusion gene expressing cells reduced the specific transcript expression by 85-90% (ABL-normalized levels were measured 24 hours after transfection). In particular, E2A-PBX1 silencing affected the EB-1 gene, which encodes for a protein that could contribute to the transformed phenotype of pre-B ALL. The detected EB-1 expression was reduced to 25%, compared to normal expression levels in non-transfected 697 cells. Furthermore, the significant decrease in the Wnt16b mRNA (and not in the Wnt16a isoform of Wnt16 gene), observed consequently to the fusion gene depletion, confirms the hypothesis of Wnt-ì16b as target of E2A-PBX1. *Conclusions.* Targeted E2A-PBX1 inhibition leads to a reduced expression of the EB-1 and Wnt16b genes, and their aberrant expression may therefore be a key-step in leukemogenesis in t(1;19)-positive pre-B leukemia.

PO-044

INCIDENCE OF DIFFERENT FUSION ONCOGENES IN ACUTE LYMPOBLASTIC LEUKEMIA (ALL) PATIENTS FROM PAKISTAN: POSSIBLE IMPLICATION IN DIFFERENTIAL DIAGNOSIS, PROGNOSIS, TREATMENT AND MANAGEMENT OF ALL

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Introduction. Acute Lymphoid Leukaemia (ALL) is the most common paediatric cancer in the world. Many chromosomal abnormalities and gene rearrangements have been found in ALL patients. Most of the chromosomal translocation, deletions, invertions lead to formation of fusion genes which act as oncogenes e.g. BCR-ABL, MLL-AF4, TEL-AML1, E2A-PBX1 AND SIL-TAL1. Different fusion oncogenes are associated with different prognosis and response to antileukemic therapy. BCR-ABL, MLL-AF4 and SIL-TAL1 oncogenes are associated with the most aggressive disease manifestation, poor prognosis and high chances of relapses. In Pakistan, most of the ALL patients have poor prognosis and relapses are also common. this study was designed know the frequency of different fusio oncogenes and to determine thier role in pronosis and differential diagnosis in ALL.*Material and Methods*. Blood samples were collected from 135 paediatric ALL patients. RNA was extracted and fusion oncogenes were detected using Reverse Transcriptase (RT) PCR protocol. Results were counterchecked using another RT-PCR protocol and by detection of corresponding chromosomal abnormalities karyotype analysis. *Results.* Out of 135 samples, frequency of different fusion oncogene was as: BCR-ABL 68(49%), MLL-AF4 12(9.1%),SIL-TAL1 7(14%) TEL-AML1 15(11.1%) E2A-PBX1 3 (01.0%) while no fusion gene was detected in 40(14%) patients.Results were 100 percent reproduced using alternative RT-PCR protocols and karyotype analysis. *Conclusion.* Incidence of different fusion oncogene in Pakistani ALL patients is different from other populations. In rest of the world, TEL-AML1 is the most common (30%) while BCR-ABL is most common in Pakistan and TEL-AML1 is only 15%. BCR-ABL, MLL-AF4 and SIL-TAL1 fusion oncogenes account for about 72% of total ALL patients which can be attributed to overall poor prognosis and low response to treatment in Pakistani ALL patients. It is hoped that these molecular genetic studies will help in future in differential diagnosis, prognosis, treatment and clinical management of ALL in Pakistan.

PO-045

INCIDENCE OF CRYPTIC CHROMOSOME DEFECTS IN ADULT B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL)

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In B-ALL the definition of the chromosome pattern is mandatory for an accurate patients prognostic stratification and for taking appropriate therapeutic decisions. Patients with either the Ph chromosome or the 4;11 chromosome translocation, which predict an unfavourable prognosis, are candidates for alternative therapeutic procedures, while those with the t(12;21), which predicts a good outcome, are submitted to standard treatments. However, despite its relevance conventional cytogenetic (CC) is not always successful in B-ALL either because of the lack of metaphases or of the presence of bad quality mitotic figures with fuzzy chromosomes. In addition, in some patients the abnormal clone may escape cytogenetic demonstration due to its kinetic features. Recently, it has been claimed that in these patients FISH, which overcomes the technical problems of CC, may identify any chromosomal defects including cryptic aberrations. Therefore, we have applied metaphase and interphase FISH to study 43 B-ALL patients with a normal chromosome pattern on CC. The aims of our study were the followings: to detect the incidence of abnormal FISH in chromosomally normal B-ALL; to evaluate whether there is a difference between CC and FISH in the ability of detecting the Ph chromosome; to establish the true incidence of some single gene defect, especially p16/INK4A deletions and AML-1 amplifications. Subsequently, FISH findings were correlated with clinico-haematological features and outcomes. FISH was carried out with the following commercial probes: LSI BCR/ABL1 dual color single fusion,

LSI TEL/AML1 ES, LSI MLL and LSI p16(9p21)/CEP 9 dual color (Vysis, Downers Grove, IL, USA). Hybridization procedures were carried out according to manufacturer's guidelines. Cut-off values were determined after having analysed two-hundred cells from ten normal controls and using a one-sided binomial distribution with a 95% confidence interval. So, the cut-off values were fixed at 10% and 6% for the BCR/ABL1 and MLL probes and at 3% for both the TEL/AML1 and the LSI p16 (9p21)/CEP 9 probes. FISH on mitotic and interphase cells showed an abnormal pattern in 19/43 (44,1%) patients. Two patients, one each with one mitotic cell presenting the Ph chromosome on CC and with one cell harbouring the BCR-ABL rearrangement on metaphase FISH, showed the translocation in all the interphase cells examined. None of our patients presented either the ETV6-AML1 translocation or any rearrangement involving the MLL gene. The most common deletions targeted the p16/INK4A gene: 3 patients showed no red spot and two green spots corresponding to the LSI p16(9p21)/CEP9 dual color probe (p16 nullisomy), 5 showed only one red spot and two green spots (p16 monomy) and the remaining two a mixture of p16 nullisomic and monosomic cells. Two patients harboured a monosomy of the ETV6 gene in 11-15% of the cells examined, while 3 others presented an amplification of the AML-1 gene in 12-16% of the cells analysed. The MLL one gene was either deleted or amplified in one patient each. From a clinical point of view p16/INK4A nullisomy/monosomy was associated with no peculiar clinico-haematological features, but the 3 patients with p16 nullisomy were unresponsive to induction chemotherapy and survived four, six and seven months. No prognostic information could be derived from the other patients with p16 monosomy. Our study shows that i) FISH is very effective in unmasking cryptic abnormalities in chromosomally normal B-ALL since it showed an abnormal pattern in 44% of our patients, ii) in some patients the presence of an abnormal cell population carrying the Ph chromosome may be suggested by CC but is more accurately demonstrated by FISH on interphase cells, iii) p16/INK4A deletion is the most common single gene defect not only in T- but also in B-ALL, iiii) the ETV6-AML1 and MLL rearrangements are extremely rare in adult B-ALL.

PO-046

THE CLINICAL VALUE OF DISEASE DE-BULKY BEFORE IMATINIB ALONE, AS POST-CONSOLIDATION TREATMENT, IN NEWLY DIAGNOSED ADULT PATIENTS WITH BCR-ABL+ ACUTE LYMPHOBLASTIC LEUKEMIA MONITORED BY Q-RT-PCR IN A SINGLE MOLECULAR BIOLOGY LABORATORY

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Nineteen adult patients aged between 21 and 59 years (median age: 46 years) with BCR-ABL+ acute lymphoblastic leukemia (ALL) were prospectively monitored by Q-RT-PCR between August 2001 to April 2006 at the molecular biology laboratory of the Haematology Unit of the University *La Sapienza* of Rome. All patients were treated according to the GIMEMA LAL 0201/A protocol, in which Imatinib alone, at the dosage of 400 mg x 2 daily for at least six months, was administered as post-consolidation in responding patients after high-dose induction and consolidation treatment. Twelve patients (63%) harboured the p190 transcript and 3 (37%) the p210 with/without the p190 isoform. At presentation, the median BCR-ABL/ABL ratio, evaluated among the entire group of 19 patients, was 198 percent (range 86 to 594; 25th to 75th percentile 110 to 350). At the beginning of the treatment with Imatinib, all patients were in 1st complete haematological remission (18 patients after the first induction and consolidation course; 1 patient after a salvage treatment). At this time point, the median reduction from baseline in BCR/ABL transcripts calculated for each individual patients was 2.1 log (range: 0 to 4.20; 25th-75th percentile: 1.67 to 2.96). In particular, 9 (47%) of the 19 cases showed a log reduction of the BCR-ABL/ABL ratio > 2.1 (p190=7 cases; p210 = 2 cases) and they were considered as good responders to the antecedent chemotherapy, whereas 10 (53%) cases (p190=5; p210=5 cases) showed a log reduction ≤ 2.1 and they were considered as poor responder patients. Respect to the type of BCR/ABL isoforms, the median reduction from baseline in BCR/ABL levels was 2.45 log (range: 0.08-4.2; 25th to 75th percentile: 1.75-3.80) and 1.67 log (range: 0.23-42.69; 25th to 75th percentile: 0.8-2.4) in p190 and p210 positive cases, respectively (p=.049; Wilcoxon rank test). During imatinib treatment, 9 (90%) of the 10 poor responders patients presented a haematological relapse versus 3 (33,3%) of the 9 good responders (p=.02). Only one out of the 7 patients in first continuous complete remission received an allogeneic stem cell transplantation while in remission. The mean time to relapse was 4 months (range: 2-6) and 14 months (range: 6-46) in poor and good responder patients, respectively. Therefore, the actuarial probability of disease free survival was 62% (range of follow-up: 6-43 months) and 10% (median follow-up 4 months; range 1-6) for good and poor responder patients, respectively (p<.0005). Similarly, the actuarial probability of survival was 72% and 0% for good and poor responder patients, respectively (p=.0002). In conclusion, Imatinib is an highly effective post-consolidation treatment for adult Ph+ ALL patients. However, the clinical outcome after imatinib was significantly affected by the level of molecular response evaluated at the beginning of the post consolidation treatment, resulting excellent for the good responders but remaining dismal for patients who responded poorly to the initial chemotherapy.

PO-047

HIGH INCIDENCE OF BCR-ABL FUSION ONCOGENE IN PAKISTANI CHILDHOOD ACUTE LYMPHOID LEUKAEMIA (ALL) PATIENTS REFLECTS ETHNIC DIFFERENCES IN MOLECULAR GENETICS OF ALL

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Introduction. Acute Lymphoid Leukaemia (ALL) is the most common malignancy in children. Chromosomal abnormalities and gene rearrangements have been found to be the genetic basis of ALL. Chromosomal translocation, deletions, invertions etc. leading to formation of fusion genes (which act as oncogenes) account for majority of ALL cases. BCR-ABL, MLL-AF4, TEL-AML1, E2A-PBX1 AND SIL-TAL1 are the most common fusion oncoges in ALL patients. BCR-ABL oncoge is associated with the most aggressive disease manifestation, poor prognosis and high chances of relapses. In Pakistan, most of the ALL patients have poor prognosis and relapses are also common. this study was designed know the frequency of BCR-ABL fusio oncogene in local paediatric ALL patients. *Material and Methods.* Blood samples were collected from 103 paediatric ALL patients. RNA was extracted and BCR-ABL oncogene was detected using Reverse Transcriptase (RT) PCR protocol. Results were counterchecked using another RT-PCR protocol and by detection of philadelphia chromosome by lymphocyte culturing and Giesma staining followed by karyotype analysis. *Results*. Out of 103 samples, BCR-ABL fusion oncogene was detected in 51(49%) samples. Results were 100 percent reproduced using alternative RT-PCR protocol and karyotype analysis. *Conclusion.* Incidence of BCR-ABL fusion oncogene in Pakistani childhood ALL patients is very high (49%) than the reports of the rest of the world. It reflects the ethnic differences in molecular genetics of ALL which can be attributed to environmental factors and lifestyle of the people in this region. Differences in anti-leukemic therapy response and survival rates have been reported between patients from different ethnic groups and recent studies using DNA expression profiles show the their genetic basis. Thus, it can be inferred that these diffferences are due to differences in incidence of BCR-ABL fusion oncoge and other molecular determinants of the ALL.

PO-048

FREQUENCY OF CHROMOSOMAL ABNORMALITIES AND CORRRESPONDING FUSION ONCOGENES IN ACUTE LYMPOBLASTIC LEUKEMIA (ALL) PATIENTS OF PAKISTAN AND ITS IMPLICATION IN DIFFERENTIAL DIAGNOSIS AND PROGNOSIS OF LEUKAEMIA

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Introduction. Leukemia is the most common male cancer in Pakistan. Acute Lymphoid Leukaemia (ALL) accounts for about 25% leukaemia cases in Pakistan. Chromosomal abnormalities and gene rearrangements are associated with ALL. Majority of the chromosomal translocation,

deletions, invertions give rise to formation of fusion transcripts which act as oncogenes e.g. BCR-ABL, MLL-AF4, TEL-AML1, E2A-PBX1 AND SIL-TAL1. These fusion oncogenes differ in disease prognosis and response to antileukemic therapy i.e TEL-AML1, E2A-PBX1 are associated with good prognosis while BCR-ABL, MLL-AF4 and SIL-TAL1 oncogenes are associated with the most aggressive disease manifestation, poor prognosis and high chances of relapses. In Pakistan, most of the ALL patients have poor prognosis and relapses are also common. This study was designed to estimate the frequency of different fusion oncogenes and to determine thier role in pronosis and differential diagnosis in ALL. *Materials and Methods.* Blood samples were collected from 135 paediatric ALL patients. Fusion oncogenes were detected using Reverse Transcriptase (RT) PCR protocol. Results were counterchecked using alternative protocols and by detection of corresponding chromosomal abnormalities. RESULTS:Out of 135 samples, frequency of different fusion oncogene was as: BCR-ABL 68(49%), MLL-AF4 12(9.1%),SIL-TAL1 7(14%) TEL-AML1 15(11.1%) E2A-PBX1 3 (01.0%) while no fusion gene was detected in 40(14%) patients.Results were 100 percent reproduced using alternative RT-PCR protocols and karyotype analysis. *Conclusions.* Frequency of different fusion oncogene in Pakistani ALL patients is different from other geographic regions. TEL-AML1 is the most common (30%) globally while BCR-ABL is most common in Pakistan and TEL-AML1 is only 15%. BCR-ABL, MLL-AF4 and SIL-TAL1 fusion oncogenes account for more than 70% of total ALL patients which can be attributed to overall poor prognosis and low response to treatment in Pakistani ALL patients. It is hoped that these molecular genetic studies will help in future in differential diagnosis, prognosis, treatment and clinical management of ALL in Pakistan.

PO-049

SURFACE THERAPEUTIC TARGET EXPRESSION IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Immunophenotyping is an essential step in the standard analysis of acute lymphoblastic leukemia (ALL) both at diagnosis and during the follow up. In particular, the pattern of expression of the differentiation antigens can be useful in four ways: 1) for lineage assignment, 2) for biphenotypic acute leukemia detection, 3) for ALL sub-classification and 4) for devising abnormal sets of markers or patterns useful for minimal residual disease monitoring. In addition, the recent introduction of monoclonal antibodies in ant-cancer therapy makes immunophenotyping a suitable tool in order to candidate patients for specific treatments. To date, similar approaches have been reserved to only few selected ALL subtypes, such as mature B-ALL. *Aim of the study.* In this study, we intended to perform the first comprehensive expression analysis of possible surface therapeutic targets in different molecular subtypes of ALL. In particular, we aimed to assess the expression pattern of CD19, CD20, CD22, CD33, and CD52, at both RNA and protein level in different ALL subtypes, by gene expression profiling and flow cytometry. *Methods.* First, in order to perform a general evaluation, we explored in silico gene expression data from 136 ALL cases (including 15 BCR/ABL1+, 20 MLL, 14 T-ALL, 18 E2A/PBX1+, 20 TEL/AML1+ and 28 of other types) studied with the HG-U133A Affimetrix GeneChip. Secondly, we analyzed the immunophenotypes of 104 ALL patients (including 34 BCR/ABL1+, 3 E2A/PBX1+, 5 MLL, 14 T-ALL, and 48 of other types) treated at our Institution from 1996 to 2005, for whom complete cytogenetic data were available. *Results.* Gene and protein expression data were substantially consistent. CD19 was highly expressed in all ALL subtypes but T-ALL (95% vs 11%, $p<0.001$); CD20 was expressed in 32% of cases without significant differences in different subgroups; CD22 was expressed in the majority of B-ALL but in only few T-ALL cases (96% vs 17%, $p<0.001$); CD33 was expressed in half of the cases (44%) including few T-ALL. Finally, CD52 was expressed in the majority of cases including T-ALL (84%). As regards the mean percentage of positive cells in individual cases, CD19 and CD22 were generally highly expressed (~ 65%), CD33 and CD52 showed intermediate values (40 and 55%, respectively), while CD20 was present at lower levels (16%). *Conclusions.* Our comprehensive analysis of surface markers in ALL showed that CD19, CD22 and CD52 are expressed in the vast majority of ALL cases (~ 85-90%), thus representing suitable therapeutic targets. No significant differences according to karyotype were recorded, while T-ALL, as expected, presented with peculiar characteristics, being CD52 the most frequently expressed molecule. CD20 was expressed in a low but still significant% cases which might benefit from specific treatment. Finally, CD33, a widely accepted target in acute myeloid leukemias, is frequently expressed (44%) and may represent an interesting target in ALL as well. Clinical trials including monoclonal antibodies are definitely warranted in ALL therapy.

This work was supported in part by COFIN 2002-2003 (Prof. M Baccarani), Centro Interdipartimentale per la Ricerca sul Cancro G. Prodi Associazione Italiana per la Ricerca sul Cancro (AIRC), AIL Bologna, Ateneo 60% (Prof. M Baccarani), and Fondazione del Monte di Bologna e Ravenna grants.

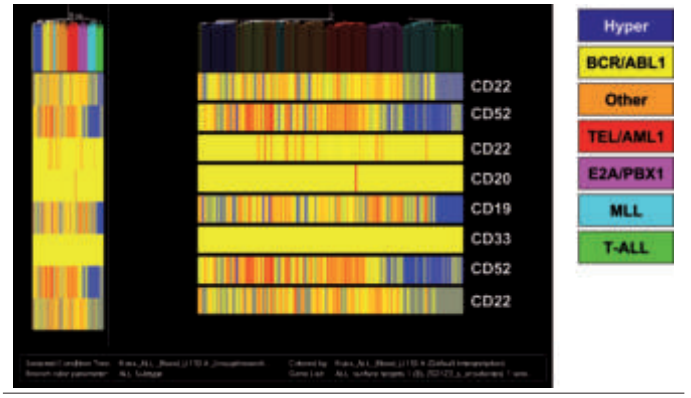

Figure 1. Gene expression analysis of 136 ALL.

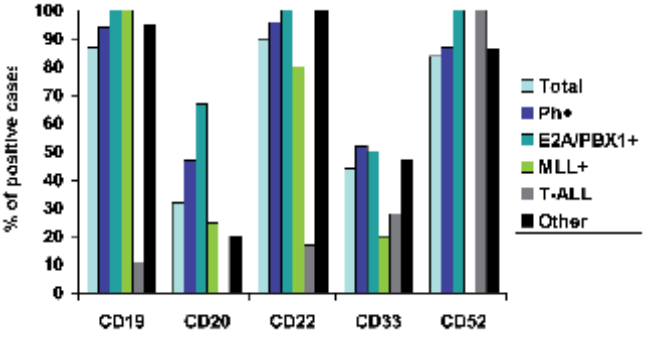

Figure 2. Markers expression by flow cytometry in 104 ALL.

PO-050

PROGNOSTIC ROLE OF MTHFR GENOTYPES IN A COHORT OF ALL PATIENTS

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Background. Several prognostic factors have been used to stratify ALL patients' risk, including clinical and biological characteristics (age, WBC count, cytogenetic or molecular aberration and more recently the kind of early response to treatment). Recently the influence of polymorphisms of different genes involved in metabolism of chemoterapic agents have been studied expecially in childhood ALL. Methylenetetrahydrofolate reductase (MTHFR) catalyzes conversion of 5,10-methyltetrahydrofolate to 5-methyltetrahydrofolate in the folic acid cycle. The C677T and A1298C MTHFR polymorphisms affect MTHFR enzyme causing a reduction of its activity, altered distribution of intracellular folate metabolites. Since MTHFR plays this important role in folate metabolism, differences in its activity due to these two gene variants might modulate therapeutic response to antifolate chemoterapeutic agents. In this study we evaluated the influence of classical prognostic factors and of C677T and A1298C MTHFR polymorphisms on time to

relapse and survival in a group of 82 ALL patients. *Patients.* Patients' characteristics were: 46/36 M/F, median age 37.35 (12-75), B-phenotype L1-2 64pts, L3 7pts and T phenotype 11, normal karyotype in 45pts and high risk karyotype in 28pts. Forty-seven patients showed WBC>10×10^9/L at diagnosis. Forty-four pts relapsed at a median follow-up of 12 months (range 1-159) and 34 pts are alive at a median follow-up of 21 months (range 1-190). *Results*. The genotypes frequencies were consistent with previous published reports. The polymorphisms' distribution among different karyotype groups was homogeneous. On univariate analysis, pts with the MTHFR C677T and A1298C variant alleles did not experience significantly increased relapse and mortality risk (chi-square test p=0.82 and p=0.59 for 677 and p=0.36 and p=0.72 for 1298). Comparison of RFS and EFS between homozygous wild type and variant patients in both 677 and 1298 polymorphisms was not significant by the log rank test (p=0.79, p=0.53 and p=0.3, p=0.57 respectively), whilst RFS and EFS were significantly decreased in the presence of high risk karyotype and age >24y (p<0.0001 and p=0.03 respectively). The Cox regression analysis containing gender, age, WBC, karyotype, phenotype and MTHFR genotypes showed an increased hazard ratio (HR) relapse and mortality in patients with high risk karyotype (p<0.001 and HR 4.33 and p=<0.0001 and HR 3.67 respectively); an increased HR mortality was demonstrated in pts older than 24 years (p<0.001 and HR 0.415). Regarding WBC count at diagnosis there was no significant correlation between WBC>10×10^9/L and outcome, whilst we found an increased risk of mortality among patients with WBC>50×10^9/L (chi-square test p=0.006). We also evaluated the correlation between MTHFR genotypes and methotrexate intolerance and experienced significantly increased toxicity among the subset of patients submitted to homogenous MTX-based maintenance regimen, thus confirming our previous data regarding the C677T variant (p=0.003) and moreover demonstrating an influence of the A1298C genotype on MTX toxicity (p=0.038). *Conclusions*. In our study we did not observe any association between MTHFR polymorphisms and relapse and survival rate in a group of almost adult ALL patients. Our data are in contrast with those from other groups which evaluated the influence of these two polymorphisms in pediatric standard risk patients. Due to the higher frequency of molecular alterations (t9;22 and t4;11), in our contest MTHFR polymorphisms per se has not enough power to influence DFS and EFS, when compared to classical risk factors, like karyotype aberrations, WBC at diagnosis and age, influencing prognosis.

PO-051

CO-EXISTENCE OF FUSION TRANSCRIPTS SIL-TAL1 AND BCR-ABL IN A PAEDIATRIC ACUTE LYMPHOBLASTIC LEUKAEMIA (ALL) PATIENT

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Acute lymphoid leukaemia (ALL) is the most common paediatric cancer. This is associated with chromosomal abnormalities leading to formation of fusion transcripts, which act as oncogenes. Each oncogenes is believed to be a different molecular entity which lead to Leukaemogenesis by different molecular pathway. Fusion oncogenes have been reported to be co-existing with other cytogenetic abnormaties but more than one fusiuon transcripts have never been reported in the same patient at the same time. Here we report co-existence of BCR-ABL[t(9;22)(q34;q11)] and SIL-TAL1 [(del 1p)] fusion genes at the same time in a paediatric ALL patient. Both fusion genes were detected by a very sensitive RT-PCR assays and confirmed by other molecular cytogenetic techniques. As both BCR-ABL and SIL-TAL1 fusion genes lead to leukaemogenesis by different pathways, such patients may be very diffucult to treat even with targetted protein tyrosine kinase inhibitors like Imatinib. Further exploration of this patient can provide very valuable information which will help not only understanding biology of leukaemogenesis but also to discovery of effective therapeutic agents.

PO-052

CXCL12-MEDIATED NF-KAPPAB AND C-JUN ACTIVATION REGULATES INTERLEUKIN 8 PRODUCTION IN HUMAN T-LYMPHOBLASTIC LEUKEMIA

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T acute lymphoblastic leukemia (T-ALL) cells arise inside the thymus and, in most cases, migrate to the bone marrow (BM) where proliferate and expand. Thymic and BM microenvironments play a fundamental role in controlling survival, proliferation, and metabolic functions of T-ALL cells. In this study we explored whether BM-derived stroma can modulate in primary T-ALL cells the production of interleukin (IL)-8, a cytokine that contributes to human cancer progression. To this aim, we performed lympho-stromal co-cultures between normal human BM stroma and primary T-ALL cells. After co-culture, we isolated T-ALL from BM cells by fluorescence activated cell sorter (FACS) and analyzed them for the presence of IL-8 mRNA. The interaction with BM-stroma induced a significant increase of IL-8 mRNA in leukemic blasts. The functional blockade of CXCL12 receptor, CXCR4, but not TNF or IL-1R, completely impaired IL-8 induction, thus indicating the involvement of CXCL12/CXCR4 axis in the BM-mediated IL-8 production. In addition, treatment of primary T-ALL cells with recombinant SDF-1 induced a significant increased production of IL-8. The induction was specific for IL-8, as IL-1, IL-6, TNF, or IL-10 production was unchanged after CXCL12 treatment. IL-8 increased expression was also confirmed at mRNA level. Finally, we showed that the activation of nuclear factor (NF)-κB and c-Jun, which represents fundamental pathway in the induction of IL-8 gene transcription, was required for the CXCL12-mediated IL-8 production. Our results implicate a novel function for CXCL12 in regulating IL-8 expression in T-ALL through the activation of the NF-kappaB and c-Jun pathways. We propose that this novel function of CXCL12 in inducing IL-8 production in leukemia cells may be relevant for the expansion of T-ALL inside the BM microenvironments.

PO-053

METHODOLOGICAL APPROACH TO MINIMAL RESIDUAL DISEASE DETECTION BY FLOW CYTOMETRY IN ADULT B-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA

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Flow cytometry is widely used for leukemia diagnosis and is suitable for routine minimal residual disease (MRD) assays. In childhood ALL flow cytometry and PCR can be highly concordant. MRD detection by flow cytometry is based on the identification of leukemia-associated immunophenotypes (LAIPs), which are not expressed by normal bone marrow (BM) or peripheral blood cells. While T-ALL blasts normally express nuclear TdT/cytoplasmic CD3 in more than 90% of cases, B-lineage ALL blasts usually share most phenotypes of normal BM B-cell precursors. Thus, the identification of LAIPs requires first that the phenotypic profiles of normal cells are fully characterized. LAIPs can be currently identified in 95% of childhood ALL cases. Fewer studies have addressed the feasibility, sensitivity, reproducibility, and prognostic significance of flow cytometric MRD detection in adult ALL. A flow cytometric approach to minimal residual disease (MRD) monitoring useful in childhood B-lineage acute lymphoblastic leukemia (ALL) is here discussed in the context of ALL in adults. We characterized adult normal BM B-cell precursors and compared the marker combinations to those expressed by adult B-lineage ALL blasts. Then, we assessed the expression stability of the identified phenotypes, and eventually the potential prognostic significance of MRD monitoring with these phenotypes. Of 64 leukemia samples analyzed, 95.3% had at least one abnormal phenotype (two or more in 57.3%) as compared to physiologic B-cell pre-

cursors in adult bone marrow. The sensitivity of the method was 1 leukemic cell among 10,000 normal cells in 16/19 experiments (84.2%). Blast phenotypes were stable in culture and at relapse, and were useful for MRD monitoring in patients. We conclude that marker combinations for childhood ALL are also applicable to adult cases.

PO-054

DETECTION OF DOUBLE FUSION TRANSCRIPTS/ ONCOGENES (BCR-ABL, SIL-TAL1) IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKAEMIA (ALL)

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Acute Lymphoid Leukaemia (ALL) is associated with many chromosomal abnormalities leading to formation of fusion transcripts. These fusion transcripts act as oncogenes, leading to leukaemogenesis. Each oncogenes is believed to be a different molecular entity and they lead to Leukaemogenesis by different molecular pathways. Many reports show the existence of fusion oncogenes with other cytogenetic abnormaties.However, more than one fusiuon transcripts have never been reported in the same patient at the same time. Here we report co-existence of BCR-ABL[t(9;22)(q34;q11)] and SIL-TAL1 [(del 1p)] fusion genes at the same time in a paediatric ALL patient. A very sensitive RT-PCR assay was employed to detect both fusion genes and findings were confirmed by other molecular cytogenetic techniques. As both BCR-ABL and SIL-TAL1 fusion genes lead to leukaemogenesis by different pathways, such patients may be very diffucult to treat even with targeted protein tyrosine kinase inhibitors like Imatinib etc. Leukemogenesis caused by BCR-ABL and SIL-TAL1 is very severe. Therefore, further exploration of these findings will provide very valuable information in understanding biology of leukaemia in patients with complex cytogenetic abnormalities.

PO-055

FIRST REPORT OF CO-EXISTENCE OF DOUBLE FUSION TRANSCRIPTS (BCR-ABL, E2A-PBX1) IN T-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA (ALL)

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Acute Lymphoid Leukaemia (ALL) is associated with many chromosomal abnormalities leading to formation of fusion transcripts, which act as oncogenes. Each oncogenes is believed to be a different molecular entity and they lead to Leukaemogenesis by different pathways. Although fusion oncogenes have been reported to be co-existed with other cytogenetic abnormaties, more than one fusiuon transcripts have never been reported in the same patient at the same time. Here we report co-existence of BCR-ABL[t(9;22)(q34;q11)] and E2A-PBX1 [(1;19)(q23;p13)] fusion genes at the same time in a paediatric ALL patient showing T-cell ALL. Both fusion genes were detected by a very sensitive RT-PCR assays and confirmed by other molecular cytogenetic techniques. T-cell ALL has also been rarely associated with BCR-ABL and E2A-PBX1 fusion genes. As both BCCR-ABL and E2A-PBX1 fusion genes lead to leukaemogenesis by different pathways, such patients may be very diffucult to treat even with targetted protein tyrosine kinase inhibitors like Imatinib. Further exploration of this patient can provide very valuable information which will help not only understanding biology of leukaemogenesis but also to discovery of effective therapeutic agents.

Chronic Lymphocytic Leukemia I

PO-056

BIALLELIC DELETION OF CHROMOSOME 13Q IN A PATIENT WITH B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA WITH RAPID CLINICAL PROGRESSION

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Chromosome analysis by cytogenetics or interphase fluorescence in situ hybridization (FISH) have identified recurring abnormalities of prognostic significance in chronic lymphocytic leukemia (CLL). Chromosomal abnormalities of 13q14 locus identified by FISH have a good prognosis when present as monoallelic deletions. Biallelic deletions of 13q14 locus have been described rarely and appear to be associated with a poor prognosis. We report a new case of a CLL patient carrying biallelic deletion of 13q associated with 11q deletion with rapid progression of disease after diagnosis. A 87 year old man referred to our institution for the documentation of severe anemia (Hb 8.3 g/dL), thrombocytopenia (PLT 90×10^6) and lymphocytosis (WBC 68.9×10^6, Ly 98%). Immunophenotyping of the lymphocytes was consistent with the diagnosis of CLL (CD19/CD5 98%, SmIg 98% MFI 21, CD23 90%, kappa/lambda 100/0%, ZAP70 24% MFI 6). The tumor clone used the VH1-69 gene segment that was unmutated (homology to germline 100%). Conventional cytogenetics was not informative. Fish analysis was performed on TPA stimulated peripheral blood lymphocytes using a multicolour probe set for deletions of chromosomes 11q23 (LSI ATM), 13q (D13S319/LSI13q34), 17p13 (LSI p53) and trisomy 12 (CEP12) and for deletion of RB1 gene (LSI 13q14)(Vysis Inc., Downers Grove, IL, USA). Slides were prepared following manufacturer protocol. For each probe at least 200 nuclei were evaluated by three different observers. Interphase nuclei showed deletions of chromosomes 11q23(96%) and 13q14(97%) comprising a mosaicism with monoallelic del 13q14.3 in 47% and biallelic del 13q14.3 in 50%. Both mono and biallelic deletions involved D13S319 locus but didn't show loss of RB1 gene. Absence of 13q14 deletion and chromosomal abnormality of 11q23 and 17p13 are significantly associated with unmutated VH genes and ZAP70 expression that are poor prognostic factors. In this case we observed the presence of a rarer biallelic deletion of 13q14 locus with unfavourable secondary anomalies and suggest that this abnormality may occur during disease progression as an indicator of clonal evolution of CLL. While this hypothesis should be validated in a greater number of cases followed over years, this case confirms that biallelic 13q deletions associate with poor prognostic markers.

PO-057

B LYMPHOCYTES IN HUMANS EXPRESS ZAP-70 WHEN ACTIVATED *IN VIVO*

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ZAP-70 is a protein tyrosine kinase initially found in T and NK cells. Recently, this important signalling element was detected in leukemic B cells from a subgroup of patients with B-cell chronic lymphocytic leukemia (B-CLL). In this study, ZAP-70 was detected in normal B cells from human tonsils, but not from peripheral blood. The cDNA sequence of B cell ZAP-70 was the same as that in T cells. Germinal centre B cells and plasma cells had a substantial proportion of ZAP-70-positive cells, while memory and follicular mantle B cells, which contain low numbers of activated B cells, expressed relatively little ZAP-70. A cell fraction of IgD-positive, CD38-positive B cells, which are comprised of many *in vivo* activated B cells, exhibited the highest levels of ZAP-70. Density gradient fractionation of tonsil B cells confirmed that ZAP-70 was not expressed by resting B cells, but was expressed by buoyant, *in vivo* activated B cells. In these B cells, the expression of ZAP-70 correlated with that of CD38 and not with that of

CD5, a hallmark of B-CLL cells. B-CLL cells are activated cells and their ZAP-70 expression reflects a normal property of activated B cells populations rather than a neoplastic aberration.

PO-058

DIAGNOSTIC POTENTIAL OF CD38 COMBINED WITH ZAP-70 EXPRESSION IN PREDICTING MUTATIONAL STATUS OF IMMUNOGLOBULIN HEAVY-CHAIN VARIABLE REGION IN 450 CHRONIC LYMPHOCYTIC LEUKEMIA CASES

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Recent advances in the diagnosis and molecular characterization of CLL permit improved prediction of disease prognosis, which could result in better management. The best-studied parameters are somatic hypermutation of the immunoglobulin heavy-chain variable region (VH), expression of the cellular proteins CD38 and zeta-associated protein 70 (ZAP-70). In this study, we analyzed the mutational status of the VH genes in CLL cells from a series of 450 cases and correlated the results with CD38 expression detected by flow-cytometry and ZAP-70 using Western blotting. Determination of Zap-70 was carried out by Western blot. For the purpose of this study, samples showing a negative and weak ZAP-70 patterns were collectively analysed. The B-CLL Ig VH gene usage and mutation was determined on cDNA according previously reported methods. The best cut-off point for CD38 or Zap-70 expression for discriminating between mutated and unmutated IgVH status was sought by constructing receiver operating characteristic (ROC) curves, which were generated by calculating the sensitivities and specificities of data at several predetermined cut-off points. Moreover, the usefulness of CD38 and ZAP expression in identifying VH mutational status was analysed according to the following standard diagnostic tests: sensitivity and specificity, positive and negative predicted values and accuracy, as well as by Kappa statistic. As a first step, we determined, by ROC curve analysis, 30% as the best cut-off value of CD38 which discriminates between mutated and unmutated cases in CLLs (area under the curve 0.758, $p<0.0001$). On the basis of standard diagnostic tests, CD38 expression, categorized by 30% cut-off value, had relatively low sensitivity (70%), specificity (77%), negative predictive (76%) and positive predictive (71%) values in anticipating VH mutational status. Moreover, Kappa statistic revealed that the agreement between CD38 expression and VH mutational status was low although significant (K=0.47, $p<0.001$). On the other hand, ZAP-70 showed very low sensitivity (57%), high specificity (89%), low positive predictive value (57%), relatively low negative predictive value (72%) and a low, although significant, K statistic (0.47, $p<0.001$). Furthermore, we combined the value of both tests to evaluate whether both variables improved diagnostic informational to that obtained from each single test in estimating VH mutational status. In this regard, we obtained the following results: sensitivity, 90%; specificity, 96%; positive predictive value, 90%; negative predictive value, 76%; k statistic 0.43, $p<0.001$). Moreover, ROC analysis was also performed to detect the optimal percentage of Ig V gene mutations capable of predicting cases with positivity of both CD38 and Zap-70. The best cut-off value was 1.9% (AUC 0.814, $p<0.0001$), which is close to the threshold (2%) used to distinguish mutated from unmutated B-CLL. Our data demonstrated that neither CD38 nor ZAP-70 by themselves had an important impact in anticipating VH mutational status. When CD38 and Zap-70 were combined, the predicting model significantly improved, meaning that the combined use of CD38 and Zap-70 could surrogate the prognostic value of VH mutational status. This information should be validated on clinical ground.

PO-059

CHROMOSOME ABNORMALITIES IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA DETECTED BY INTERPHASE FLUORESCENCE IN SITU HYBRIDIZATION: CORRELATION WITH BIOLOGICAL FEATURES.

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Background. B-CLL has a variable history with respect to time to progression and response to standard cytotoxic therapies. FISH interphase cytogenetic analysis is superior to standard karyotype analysis in identifying known abnormalities and is able to reveal chromosome changes in the majority of B-CLL samples.[1,2] The most common abnormalities are del(13)(q14.3), del (11)(q22-23), trisomy 12 and del(17)(p13.1). Del(11q) and del(17p) are definitely associated with a bad prognosis.[1] Some Authors have correlated FISH features with other biological parameters, including sex, age, Rai stage, IgVH mutational status and CD38 expression.[3] We tried to establish such correlations in a series of patients coming from several Centers in Romagna. *Materials and Methods.* FISH was carried out using VYSIS probes for each of the above mentioned abnormalities.

Table 1.

	NO Abn.	*Del(17p)*	*Del(13q)*	*Del(11)*	*Del(13q)+Del(11q)*	*Tri(12)*
N° Pts	37	5	27	6	4	7
M/F	21/16	4/1	19/8	5/1	4/0	4/3
Age						
(extr.)	[37-79]	[55-86]	[34-75]	[55-83]	[49-66]	[36-70]
(median)	58	66	58.5	70	58	62
Ly/mm^3						
(extr.)	[0.6-84.0]	[7.5-47.7]	[7.1-78.2]	[17.4-40.9]	[7.94-49.2]	[6.3-32.0]
(median x10^3)	10.2	36.7	18.4	15.8	17.2	9.0
CD38+ (%)						
(extr.)	[0-46]	[1-94]	[0-36]	[4-76]	3	[3-70]
(median)	5	6	3	40	3	40
LDH (U/mL)						
(extr.)	[275-415]	[179-402]	[239-485]	[293-355]	[228-302]	[300-578]
(median)	309	330	321	346	252.5	413
RAI stage						
0	26		19	2	1	4
I	6		4	2	1	1
II	4	2	4	1	1	2
III	1					
IV		3		1	1	
TREATED						
yes/no	9/19	2/2	9/15	4/0	3/1	2/5
no data	9	1	3	2		
Time to Ly number duplication (mos)						
(extr.)	[0-56]	[7-11]	[3-44]	[7-37]	[9-14]	[8-24]
(median)	14.5	9	16	13.5	12	8
Time to treatment (mos)						
(extr.)	[0-112]	[18-56]	[0-62]	[8-40]	[10-18]	[10-18]
(median)	16	37	31	28	14	69

Results. Up to now, 86 B-CLL patients have been studied. 56 were males and 28 were females, their age ranged from 34 to 86 years. FISH results are shown in Table 1. *Conclusions.* Preliminary data seem to confirm the results of the literature, in particular, pts with del(17p) show a higher lymphocyte count and a higher Rai stage, with respect to the other cytogenetic groups. The work is still in progress.

References

1. Dohner H et Al. N Engl J Med 2000; 343:1910-6.
2. Cuneo A. et Al. Cancer Res 1997;57:1144-50.
3. Dewald GW et Al. Br J Haematol 2003;121:287-95.

PO-060

GENE EXPRESSION PROFILING IN B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA REVEALS DIFFERENTIAL EXPRESSION LEVELS OF EDAG AND ANGIOPOIETIN-2 GENES BETWEEN UNMUTATED AND MUTATED PATIENTS

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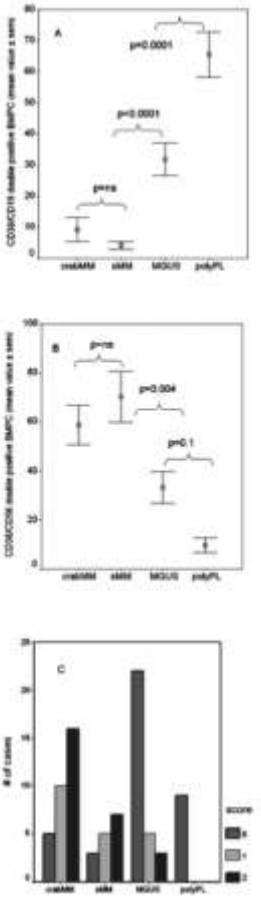
B-cell chronic lymphocytic leukemia (B-CLL) shows a high heterogeneity in the clinical course, spanning from aggressive to completely indolent behaviour. To identify the correspondent gene expression variability, we selected 29 well-defined, newly diagnosed B-CLL patients and we hybridized them on ink-jet printed microarrays comprising about 20,000 oligonucleotide probes. The mutational status of IgVH genes was examined in all 29 CLL cases. Using the classical homology cut off value of 98%, 14 patients (48.3%) had mutated VH genes (M-CLL group) and 15 (51.7%) had unmutated VH sequences (UM-CLL group). As previously reported, different immunoglobulin gene segments revealed a biased usage related to the mutational immunoglobulin VH gene and poor prognosis was associated with the unmutated subset. Firstly, using 4 different unsupervised clustering algorithms to analyze gene expression data, we identified two robust CLL clusters. Altered expression of genes mainly involved in protein biosynthesis and oxidative phosphorylation differentiated the two main CLL subgroups. These B-CLL biological clusters were not associated with Ig mutational status. Furthermore, we applied another unsupervised algorithm after the exclusion of the genes characterizing the previously identified CLL subsets identifying two new CLL clusters, showing a clear association to the Ig mutational status. In order to identify how the immunoglobulin status could affect the levels of specific genes, we applied supervised clustering based on differences in the IgVH mutation status.



Differentially regulated genes were revealed at a significant level of 0.01, yielding 128 genes. Of note, we observed in unmutated CLL cases a down-regulation of genes belonging to B-cell- and antibody-mediated immunity and immunity/defence categories. Moreover, genes involved in Wnt signalling, integrin signalling, inflammation mediated by chemokine and cytokine signalling pathways as well as in T cell activation were found down-regulated in unmutated CLLs. The analysis identified EDAG as the best discriminating gene between M- and UM-CLLs. Interestingly, EDAG regulates the proliferation and differentiation of hematopoietic cells and apoptosis resistance through the activation of NF-κ B. Again, we found the high expression in UM-CLL of angiopoietin 2 (ANGPT2), inducer of angiogenesis. We evaluated the mRNA levels of EDAG and ANGPT2 in a large cohort of both unpurified and CD19+ purified CLL samples as well as in some cases of LMA, LLA, LMC and in normal PBMCs and CD19+ selected cells, using Real-Time PCR. Then, we analyzed the correlation between EDAG and ANGPT2 mRNA levels as well as their association with immunoglobulin mutational status, haematological parameters and clinical outcome of patients.

PO-061

SIGNIFICANCE OF SERUM INTERRLEUKIN-16 LEVELS IN PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Interleukin-16 (IL-16) was described originally as a lymphocyte chemoattractant factor that is generated from mitogen antigen-stimulated human peripheral blood mononuclear cells. Subsequent studies found that IL-16 is produced by lymphocytes, mast cells, dendritic cells, epithelial cells, fibroblasts and eosinophils and induces chemotaxis via direct interaction with CD4 molecule as its natural ligand in CD4-expressing cells such as T cells, eosinophils, mast cells, dendritic cells and monocytes. A subset of CD4+ and CD8+ clones could utilize IL-16 levels as growth factors after previous mitogenic activation, but for the CD4+ subset IL-16 responses were significantly higher. Cytokine-dependent proliferation was higher for the CD4+ than for the CD8+ clones in the presence of both IL-16. The impact of IL-16 on T cell apoptosis is also discussed. In the present study, we evaluated the circulating IL-16 in chronic lymphocytic leukaemia (CLL) patients and examined its relationship with other markers of disease activity and survival profiles. *Materials and methods.* The study population included 52 newly diagnosed patients with CLL. Their median age was 64 years (range 43-87). Patients were clinically staged according to the Rai system and distributed as follows : stage 0 14, stages I-II 28 and stages III-IV 10. Routine laboratory studies consisted of complete blood count with differential, platelet count and blood chemistry including β-2microglobulin (β2microglobulin) and lactate dehydrogenase (LDH), as well as immunonophenotyping to establish the diagnosis of typical CLL. Serum concentrations of IL-16 was measured by standard quantitative sandwich ELISA (Quantikine) kits, according to manifactures instructions. *Results.* Serum levels of IL-16 were significantly higher in comparison to controls (21) (p<0,004). A significant difference was observed in the IL-16 concentrations at different disease stages with elevated levels at stage III-IV compared to stages 0 (p<0,0005). As far as clinico-hematological variables reflecting tumor mass are concerned, a significant correlation was found between IL-16 and white blood cells (WBC) (r=0,677), absolute peripheral blood lymphocytosis (PBL) (r=0,684) and LDH (r= 0,595). Interestingly, the linear correlation between serum levels of IL-16 and Syndecan-1 (r=0,681) suggest an involvement in the processes leading to anti-apoptosis. No correlation was found between concentrations of IL-16 and CD4 and CD8, nevertheless the absolute number of lymphocytes in the CLL was increased. In conclusion, a high serum IL-16 level detected in newly diagnosed CLL patients and its correlation with known factors of disease activitys show may play a role in mechanisms of disease-progression of CLL.

PO-062

CLL T CELLS HAVE REDUCED EXPRESSION OF EARLY TCR SIGNALING MOLECULES CD3 EPSILON AND LAT: IMPLICATIONS FOR T-CELL DISFUNCTIONS IN CLL

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Chronic lymphocytic leukemia (CLL) is a hematological disease characterized by the accumulation of circulating monoclonal CD5+ B-cells Besides functional abnormalities within the neoplastic B cells, several abnormalities, have also been demonstrated in the non-neoplastic T cells of CLL. Several studies reported a disfunction of the immune system in cancer patients. A down-regulation of TCR-related CD3 zeta protein and p56 Lck kinase was described in T cells from patients with ovarian cancer, colorectal cancer, and renal cell carcinoma, suggesting that immune cell deregulation may result from impairment of cell signal transduction. A decreased level of CD3 zeta protein was also found in hematological diseases such as B-cell lymphomas and Hodgkin's lymphomas. Moreover a recent study reported a decrement of CD3 zeta, p56 Lck, ZAP-70 and PI3-kinase in T cells of multiple myeloma. A reduction of CD3 zeta levels was also reported in CLL. However, little else is known about other molecular defects of the T cell signalling pathways. We have investigated cases of CLL for the expression levels of the proteins involved in the early TCR signalling events. CD3+ T cells of 7 B-CLL patients and 4 healthy donors were purified using T cell isolation kit II from Milteny Biotec and lysed in 1% Triton X-100. Subsequently, equal amounts of protein for each sample were analyzed by Western blotting, to evaluate the expression of the following molecules: CD3 epsilon, p56Lck, ZAP-70, LAT, PLC γ1. In our study we observed a different pattern of expression among these molecules in the CLL cases. In fact, apart from PLC γ 1 and Zap-70, that were equally expressed in T cells of B-CLL and normal healthy donors, the CD3 epsilon subunit of the TCR complex and p56 Lck tyrosine kinase were reduced in 4/7. More interestingly, LAT (linker for activation of T cells) expression appeared strongly reduced in all but one CLL. LAT is an adaptor molecule which, even if lacking in enzymatic activity, plays a critical role in T cell signalling acting as a plasma membrane scaffold and facilitating the assembly of numerous signalling complexes. In conclusion these data show a decrement in the levels of this crucial protein in T cells of B-CLL, and suggest important implications for the understanding of the molecular mechanisms underlying T cell disfunctions in CLL.

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PO-063

RIDUZIONE DELL'ANGIOGENESI MIDOLLARE DOPO TERAPIA DI CONSOLIDAMENTO CON BASSE DOSI DI ALEMTUZUMAB IN PAZIENTI CON LEUCEMIA LINFATICA CRONICA

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We evaluated microvessel area of bone marrow (BM) samples from 20 patients with advanced chronic lymphocytic leukemia (CLL) who received at least 8 weeks after the end of treatment with fludarabine subcutaneous alemtuzumab, three times weekly for 6 weeks, at a dose of 10 mg. The rate of CR increased from 55% after fludarabine to 90% after alemtuzumab consolidation (p=0.03; Fisher's exact test). In keeping with hematological responses, significant changes of BM angiogenesis were observed. The assessment of microvessel area carried out at the starting of therapy, after fludarabine and at the end of therapy with alemtuzumab, respectively, showed a continuous decrease in the extent of microvessel area (p=0.002). After consolidation with alemtuzumab, 13 out of 20 (65%) patients changed from a monoclonal to a polyclonal pattern of IgH. A separate evaluation carried out in patients with a persistent monoclonal IgH pattern and in patients who changed to a polyclonal pattern of IgH after therapy with alemtuzumab showed a significant reduction of BM microvessel area only in the latter (p=0.0002). Finally, a significant decrease of the extent of BM angiogenesis was observed among patients who had received a cumulative dose of alemtuzumab higher than median (i.e.184 mg) (p=0.0001) while the same did not apply for those who had received cumulative dose of alemtuzumab lower than median (p=0.127). In conclusion, a decrease in BM vascularity was observed after treatment with alemtuzumab. Such a finding reflects either molecular response or cumulative dose of alemtuzumab. These observations lend support to the anti-angiogenic role played by alemtuzumab in CLL.

PO-064

HAIRY CELL LEUKAEMIAS (HCL) WITH UNMUTATED V-GENES HAVE A POORER RESPONSE TO SINGLE AGENT 2CDA THAN HCL WITH MUTATED V-GENES.

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Hairy cell leukemia (HCL) is a rare B-cell neoplasm highly responsive to purine analogues (2CdA or DCF) or interferons as single agents, and only a minority are refractory. Patients who obtain any response (either complete or partial) tend to have survivals as normal healthy subjects and/or will benefit from repeating the treatments in case of relapse. Conversely, the minority of patients who do not respond to one of the drugs often do not respond to the others and have a poor prognosis. We have recently observed that the majority of HCL mutated VH genes, while a minority has unmutated VH genes. In the most common B-cell neoplasm chronic lymphocytic leukemia (CLL), VH gene status has prognostic impact and correlates with progression, treatment-response and surivival. In the process of identifying prognostic parameters of responsiveness to 2CdA, we prospectively investigated the VH and VL genes expressed by the tumor cells and response to treatment in patients receiving subcutaneous 2CdA. In newly diagnosed HCL requiring treatment, enrolled in an Italian multicenter trial (ICGHCL2004), peripheral blood mononuclear cells were obtained prior to treatment, and the expressed tumor VH and VL genes were identified by PCR and cloning. Tumor sequences with >98% homology to germline VH and/or VL genes were defined as *unmutated*. Patients received 0.1 mg/kg subcutaneous 2CdA for 5 or 7 consecutive days and responses were evaluated by immunohistochemistry of trephine bone marrow biopsies 2 and/or 6 months after the end of treatment. Of 45 patients recruited, 16 patients were evaluable for response. Definition of response was according to consensus resolution criteria. We observed that 12/16 patients responded to treatments (10 CR, 3 PR), while 3/16 patients demonstrated refractory or progressive disease. Leukocytosis was observed in 2/3 refractory, but also in 1/11 responsive patients. Most remarkably, the 3/3 refractory HCL shared the common feature of expressing unmutated VH and VL genes, in contrast to the responsive patients that all carried mutated VH and/or VL genes. From our series, there are indications that mutational status may relate with tumor burden (leukocytosis) and, more importantly, with response to 2CdA. Overall, the interim data suggest that HCL patients with unmutated VH genes may not benefit from single agent subcutaneous 2CdA and provide elements to build new clinical trials with combined strategies in cases of refractory/non responsive HCL where the immunogenenetic tumor profile is provided.

PO-065

IMMUNOPHENOTYPIC AND IMMUNOGENETIC ANALYSIS PROVIDE NEW DISTINCTIVE FEATURES OF HAIRY CELL LEUKAEMIA VARIANT

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Hairy cell leukemia variant (HCLv) is a rarest B-cell neoplasm, that distinguishes from HCL for leukocytosis, morphology and lack of moncytopenia, easy aspiration of bone marrow, and generally absence of CD25 expression on the tumor cells. We have recently observed that typical Hairy cell leukemias characteristically co-express multiple Ig isotypes, lack the memory B-cell marker CD27 and that the majority express mutated VH genes and only a minority have unmutated VH genes. In the process of identifying additional distinguishing features between HCL and HCLv, we determined expression of IgM, IgD, IgG and IgA surface isotypes and CD27 memory B cell marker in flow cytometry, and investigated the expressed tumor VH genes in 3 cases of HCLv. As observed

in typical HCL, all HCLv expressed multiple Ig isotypes (IgD IgG in case 1, IgM IgG IgA in case 2, and IgG IgA in case 3) on the surface of tumor cells, indicating mechanisms of ongoing class switch recombination as for typical HCL. Analysis of tumor VH genes revealed that all cases used VH3 family (3-30, 3-07 and 3-23 respectively). Two of three cases carried mutated VH genes, while case 1 carried completely unmutated VH genes (100%, 92,22% and 97,42% homology to germline respectively). Analysis of tumor VL genes confirmed mutational status of VH genes in two cases, since the expressed VL genes (lambda V2-14, kappa V1D-33) were mutated (97,03 and 95,83% homology to germline, respectively). Curiously, case 1, that had completely unmutated VH, expressed the kappa light chain VK1-5, that was mutated (95,02%), suggestive of secondary recombination of the VH gene occurring in the periphery, likely after antigen encounter. On the other hand, in contrast to what observed in typical HCL, CD27 was clearly expressed on the tumor cells of all 3 HCLv. Analysis of Ig isotypes and somatic mutation in our cases of HCLv reveals ongoing events of isotype switching and arrest of the neoplastic cell after antigen encounter, as observed in typical HCL. The new observation of CD27 expression in HCLv reveals a new feature distingushing variant from typical hairy cell leukemias, and provides implications for the different biology of this two types of B-cell tumor.

PO-066

ANALYS OF EXPRESSED IMMUNOGLOBULIN HAEVY CHAIN GENES IN LYMPHOPROLIFERATIVE DISEASE

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During recent years it has become evident that B-cell origin display preferential immunoglobulin heavy chain gene usage. Biased immunoglobulin heavy variable (VH), diversity (D), and joining (JH) gene usage has been reported in different entities of B-cell lymphoma, B-lineage acute lymphoblastic leukaemia (ALL) and in B-cell chronic lymphocytic leukaemia (CLL). This non random usage of individual VH, D, and JH genes suggest that the immunoglobulin structure may play a role in leukaemia /lymphoma development, possibly through unknown antigen stimulation. We examined the immunoglobulin (Ig) heavy chain rearrangements used by leukaemia B-cells of 74 unrelated patients. *Methods.* Peripheral blood (PB) or bone marrow (BM) samples from 46 B-ALL and 28 CLL patients were obtained at diagnosis. DNA was prepared using standard protocols and amplification of rearranged Ig heavy chain genes was performed using consensus VH/JH primers as described in BIOMED 2. Clonal PCR products were directly sequenced (BigDye Terminator Cycle Sequencing Kit, Applied Biosystems) and analyzed by an automated sequencer (ABI PRISM 310 Applied Biosystem). Sequences were aligned to Ig sequences using the IMGT database and the IGBlast search. *Results.* We examined the immunoglobulin genes used by leukemia cells of 28 patients with CLL. We found 21 cases with 1 rearrangement (75%), 7 cases with 2 rearrangements. The mutational status of Ig was analyzed: 31.6% of clones were mutated 68.4% unmutated. In the 7 cases who displayed more than one IgH rearrangement, 4 cases had only unmutaded VH gene rearrangements, whereas 3 cases had one mutated and one unmutated VH gene rearrangement. The most common variable gene family used was VH1 (42.8%), followed by VH3 (39.2%), VH4 (21.4%) and VH6 (2.8%); no VH5 or VH2 were found. We find a preferential use of the VH1 family member VH1-69 (21%) exclusively in unmutated CLL and of VH3-21 (14.2%) in both mutated and unmutated status. In ours series the joining gene family JH6 occurred most commonly (40%), followed by JH4 (37.1%). The most common diversity used gene was D3. Diversity segments were not used preferentially with any VH or JH gene. A total of 52 IgH sequences were identified from 46 patients with B-ALL: a single IgH sequence was identified in 41 cases (89%) whereas in 5 cases (11%) biclonal or oligoclonal rearrangements were found. VH3 (36.5%) VH4 (21.1%) and VH1 (17.3%) amounted to 75% of rearranged families. Usage of D2 and D3 families was most prominent (40.3% and 30.7% respectively). JH6 occurred most commonly and was found in 51.9% rearrangements followed by JH4 (26.9%). *Discussion.* We analyzed VH gene segment usage during VDJ rearrangement in B-cells from adult ALL and CLL population. As expected from germline pattern seen in normal BM and PB, the largest number of clones identified was VH3, VH4 or VH1. However we observed an overrepresentation of VH1 in CLL compared with peripheral B lymphocyte and a significant privileged usage of VH6 rearrangement in adult ALL. Some IgV genes (VH5) appeared to be suppressed when their frequencies was compared to the normal cell repertoire. These findings, in agreement with others, provide evidence that, independently of geographic origin, Ig expressed in lymphoproliferative disease are highly selected and not representative of Ig expressed by naïve B cells.

PO-067

SPONTANEOUS APOPTOSIS AND PROLIFERATION IDENTIFY DIFFERENT PROGNOSTIC SUBSETS IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA.

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Dysfunctional apoptosis and cell cycle are important reasons for the clinical enigma, that B-cell chronic lymphocytic leukemia (B-CLL) can not yet be cured with conventional chemotherapy. In B-CLL, malignant cells seem to be arrested in the G0/early G1 phase of the cell cycle, and inhibition of spontaneous apoptosis and upregulation of the anti-apoptotic protein bcl-2 define clinical prognosis. However, increasing evidence exists that disease progression relies upon cycling B-CLL cells: a proliferating pool of cells has been described in lymph nodes and bone marrow and might feed the accumulating pool in the blood. Moreover, the lack of immunoglobulin (Ig) VH gene mutation also has been shown to predict a rapid disease progression (DP) and an inferior overall survival (OS) (Damle, Hamblin, 1999). B-CLL cells that use non-mutated IgVH genes express ZAP-70 RNA, which encodes ZAP-70, a 70-kDa protein tyrosine kinase, associated both with an enhanced B cell receptor signaling and with an early DP risk in B-CLL (Del Principe, 2006). Moreover, the today availability of rapamycin or proteasome inhibitors effective against proliferating B-CLL cells and bcl-2 antisense oligonucleotides prompted us to evaluate the real impact of proliferation and apoptosis pathways on B-CLL prognosis. The primary aims of our study were: 1) to determine progression-free survival (PFS) upon apoptosis/proliferation subgroups and ZAP-70 expression; 2) whether apoptosis/proliferation could predict varied outcome within ZAP-70 subgroups; and finally 3) whether ZAP-70 and apoptosis/proliferation groups were independent prognostic factors. Therefore we investigated 265 pts, median age 64 years (range 37-84), 136 males and 129 females. With regard to modified Rai stages, 87 patients had a low stage, 170 an intermediate stage and 8 a high stage. ZAP-70 was quantified by a multicolor flow cytometric method fixing a cut-off value of 20%. Bcl-2 was determined by flow cytometry, dividing mean fluorescence intensity (MFI) of CD19+B-CLL cells / MFI of T-cells (Bcl-2B/T). The threshold was set at the median value >1.6. Transferrin receptor (CD71) was used as a measure of the proliferation and the threshold was set at the median value >8%. Combining Bcl-2B/T with CD71 (Bcl2CD71) we enucleated three subgroups: 1) Bcl2CD71- [106 pts] with low proliferation (CD71 <8%) and high apoptosis (Bcl-2B/T <1.6); 2) Bcl2CD71+ [49 pts] with high proliferation (CD71>8%) and low apoptosis (Bcl-2B/T >1.6); and 3) Bcl2CD71+/- [110 pts] with low proliferation and low apoptosis or with high proliferation and high apoptosis. ZAP-70+ B-CLL patients were 95/265 (36%). In 111 studied pts ZAP-70 expression and Ig V gene mutational status were significantly correlated (p<0.00001). Furthermore, we found significant associations either between lower ZAP-70 and lower Bcl-2B/T index (p=0.001) or lower ZAP-70 and Bcl2CD71- (p=0.002), confirming that low levels of ZAP-70 were characterized by high apoptosis and low proliferation. With regard to clinical outcome, a significant shorter progression-free survival (PFS) was observed in ZAP-70+ pts vs ZAP-70 negative pts (0% vs 58% at 13 years; p<0.00001) and in Bcl2CD71+ pts vs Bcl2CD71- pts (10% vs 56% at 12 years; p<0.00001). The Bcl2CD71+/- subgroup showed an intermediate outcome (30% at 12 years). To further explore the prognostic impact of Bcl2CD71 index, we investigated its expression within ZAP70+ (95 pts) and ZAP70- (170 pts) subsets. As a matter of fact, Bcl2CD71 was not able to identify prognostic subsets within ZAP-70+ pts, because all these cases presented a shorter PFS without significant differences. On the other hand, this index

identified subsets at different PFS within the ZAP-70 negative subgroup (73% for Bcl2CD71- pts vs 29% for Bcl2CD71+ at 12 years, p=0.00009). In multivariate analysis of PFS, in which age, Rai modified stages, CD38, soluble CD23 (sCD23), lymphocyte doubling time (LDT), Bcl-2CD71 and ZAP-70 entered, ZAP-70 (p=0.00005), LDT (p=0.006), Rai modified stages (p=0.03) and sCD23 (p=0.01) resulted to be independent prognostic factors. Therefore, ZAP-70 was confirmed as the most important indipendent prognostic factor with regard to PFS. However, our apoptotic/proliferative index (Bcl2CD71), performed by flow cytometry, was very useful to identify pts at different progression rate within the ZAP-70 negative subgroup. Since the ZAP-70 negative subset represents a large and heterogeneous B-CLL population with a variable progression, other biological factors, such as the amount of apoptosis and the proliferative rate, have to be added in order both to identify early progressive pts and to take timely accurate therapeutic decisions.

PO-068

IL-21 AND IL-15 DISPLAY OPPOSITE EFFECTS ON CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) B CELLS: APOPTOSIS VERSUS SURVIVAL.

De Totero D,[1] Meazza R,[3] Capaia M,[1] Zupo S,[2] Fabbi M,[1] Cutrona G,[2] Matis S,[2] Colombo M[2] Gobbi M,[4] Balleari E,[4] Pierri I,[4] Azzarone B,[5] Ferrarini M,[2] Ferrini S[1]

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Interactions between CLL and microenviroment are critical in expansion and survival of the neoplastic B cell clone, and cytokines have a relevant role in this process. IL-21, a member of the common-γ chain cytokine family, may mediate proliferation or apoptosis of normal B cells, depending on their functional state. We recently showed that IL-21R is frequently expressed on CLL B cells and further up-regulated by CD40 triggering. Moreover IL-21 is capable of inducing apoptosis of CLL B cells with more evident effects in CD40-activated cells. IL-21 induced Jak-1 and -3 auto-phosphorylation and tyrosine phosphorylation of STAT-1,-3, and -5. Apoptosis triggered by IL-21 involved caspase-8 and -3, cleavage of Bid to t-Bid, and cleavage of PARP and of p27/Kip1. In addition IL-21 counteracted the mitogenic activity and the anti-apoptotic effects of IL-15, sharing with IL-21 the common γ chain capable of transducing signals. At the same time exposure of CD40 activated cells to IL-21 consistently reduced CD23 surface expression in viable CLL cells while IL-15 displayed an opposite effect with increase of CD23 molecule. Our data demonstrate that IL-15 and IL-21 are critical in regulating growth of the neoplastic B cell clone. Studies on signaling pathways triggered after IL-21 or IL-15 treatment, which lead to apoptosis or, on the contrary, to survival are currently under investigations.

PO-069

FK506 RESCUES TGF-β RESPONSE IN B-CLL

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B Cell Chronic Lymphocytic Leukemia (BCLL) is a disorder characterized by an accumulation of mature B cells in peripheral blood, due to decreased apoptosis. BCLL cells display progressive loss of response to Transforming Growth Factor (TGF)-β, a tumor suppressor cytokine which inhibits cell growth by promoting cellular differentiation and apoptosis. FK506 binding protein (FKBP) 12 acts as a negative regulator of TGF-β receptor internalization, thereby inhibiting signaling. TGF-β receptor has a kinase activity: after ligand binding it phosphorylates Smad2 and Smad3 which associate with Smad4, then, Smad complex translocates into the nucleus where it is able to activate transcription. Recent findings of an over-activation of TGF-β signaling in fibroblasts from FKBP12-knockout mouse prompted us to investigate if the immunosuppressant FK506, the canonical ligand and inhibitor of FKBP12, can activate TGF-β signal in BCLL cells. We found that FK506 induced apoptosis in 70% of BCLL samples at doses comprised between 1 and 100 ng/mL, whereas the cyclophilin-A inhibitor cyclosporin did not display the same effect. This data suggests that the mechanism responsible for immunosuppression, i.e. calcineurin inhibition, was not involved in apoptosis induction. The apoptotic events induced by FK506 were preceded by decrease of Bcl-2 and Bcl-xL levels and mitochondrial membrane depolarization, similarly to what occurs after TGF-β signaling activation. After 24-40 hours, phosphatidyl-serine appeared on the outer leaflet of plasmamembrane, as demonstrated by annexin V staining, indicating the cell was dying. To assess if FK506 activated TGF-β signaling, we investigated the activation of Smad proteins. Western blot assay was performed to measure phospho-Smad levels, whereas flow cytometry analysis of isolated nuclei, stained with anti-Smad 4 antibodies, allowed us to investigate Smad nuclear translocation. Our data showed that FK506 induced Smad-phosphorylation and nuclear translocation after 180' of incubation, indicating the activation of the TGF-β signaling. These findings suggest that FK506 may rescue TGF-β response in BCLL with implications in the prognosis of this disease.

PO-070

FK506 RESCUES TGF-β RESPONSE IN B CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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B Cell Chronic Lymphocytic Leukemia (BCLL) is a disorder characterized by an accumulation of mature B cells in peripheral blood, due to decreased apoptosis. BCLL cells display progressive loss of response to Transforming Growth Factor (TGF)-β, a tumor suppressor cytokine which inhibits cell growth by promoting cellular differentiation and apoptosis. FK506 binding protein (FKBP) 12 acts as a negative regulator of TGF-β receptor internalization, thereby inhibiting signaling. TGF-β receptor has a kinase activity: after ligand binding it phosphorylates Smad2 and Smad3 which associate with Smad4, then, Smad complex translocates into the nucleus where it is able to activate transcription. Recent findings of an over-activation of TGF-β signaling in fibroblasts from FKBP12-knockout mouse prompted us to investigate if the immunosuppressant FK506, the canonical ligand and inhibitor of FKBP12, can activate TGF-β signal in BCLL cells. We found that FK506 induced apoptosis in 70% of BCLL samples at doses comprised between 1 and 100 ng/mL, whereas the cyclophilin-A inhibitor cyclosporin did not display the same effect. This data suggests that the mechanism responsible for immunosuppression, i.e. calcineurin inhibition, was not involved in apoptosis induction. The apoptotic events induced by FK506 were preceded by decrease of Bcl-2 and Bcl-xL levels and mitochondrial membrane depolarization, similarly to what occurs after TGF-β signaling activation. After 24-40 hours, phosphatidyl-serine appeared on the outer leaflet of plasmamembrane, as demonstrated by annexin V staining, indicating the cell was dying. To assess if FK506 activated TGF-β signaling, we investigated the activation of Smad proteins. Western blot assay was performed to measure phospho-Smad levels, whereas flow cytometry analysis of isolated nuclei, stained with anti-Smad 4 antibodies, allowed us to investigate Smad nuclear translocation. Our data showed that FK506 induced Smad-phosphorylation and nuclear translocation after 180' of incubation, indicating the activation of the TGF-β signaling. These findings suggest that FK506 may rescue TGF-β response in BCLL with implications in the prognosis of this disease.

Chronic Lymphocytic Leukemia II

PO-071

THE RESPONSE TO SURFACE IGM AND IGD CROSS-LINKING DEFINES DIFFERENT GROUPS OF B CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

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The present study investigated the response of the malignant cells from 184 B-CLLs cases to surface IgM or IgD cross-linking. The cases were grouped according the type of response observed and correlations with the expression of prognostic markers and clinical outcome were recorded. Three groups of B-CLL cells were defined by the response to surface IgM stimulation. In one group (101 cases) the cells responded by undergoing apoptosis (i.e > 20% increase over spontaneous apoptosis), in another group (8 cases) there was inhibition of apoptosis (i.e.reduction of spontaneous apoptosis by at least 20%), while in a third group (75 cases) no response was observed. Analysis of prognostic markers revealed more numerous CD38-positive cases (but not of ZAP-70 positive or mutated cases) among the B-CLL induced into apoptosis. Three groups of B-CLL also were defined by the response to stimulation through surface IgD. In one group (74 cases), the spontaneous apoptosis was inhibited by at least 20%, in another group (12 cases) there was increased by at least 20% after IgD cross-linking, while in a third group (98 cases) no response was recorded. No statistical correlations was found between prognostic marker expression and type of response to surface IgD cross-linking. When the responses to IgM and IgD cross-linking were analyzed together, the following B-CLL groups were found: A) B-CLLs that responded to IgD cross-linking by inhibition of apoptosis and did not respond to IgM cross-linking [26/184] cases (14%). This cluster was predominantly CD38-negative (81%), VH genes mutated (76%), and ZAP-70 negative-weak(84%) B) B-CLLs which responded to IgD cross-linking by inhibition of apoptosis and to IgM cross-linking by apoptosis induction [43/184 cases (23%)]. These cases were predominantly VH genes unmutated (73%) and ZAP-70 strong (54%) while approximately 50% of cases were CD38 positive. C) B-CLLs which did not respond to IgD cross-linking but responded to IgM cross-linking by apoptosis induction [49/184 cases (27%)]. These were mainly CD38-negative (65%) VH genes mutated (69%) and ZAP-70 negative-weak(78%). D) B-CLLs which did not respond significantly to both stimuli [74/184 cases (26%)]. Among these cases there was an equal distribution of CD38 and ZAP-70-positive or negative cases, although they were predominantly VH genes unmutated (63%). The distribution in the expression of prognostic markers in the above groups was statistically significant (p=0.002 for ZAP-70; p=0.04 for CD38; p<0.0001 for VH mutational status). Finally, 77 B-CLLs could also be evaluated for clinical outcome. After a median follow-up of 44 months (range 2-377), cases belonging to group A experienced a statistically significant longer treatment free survival (153 months) as compared to group B (36 months), group C (46 months) and group D (13 months, p=0.04). Collectively, these data indicate that response to surface Ig stimulation may influence the progression of disease and can be taken as a further factor for determining the prognosis.

PO-072

DYNAMIC MODIFICATIONS OF THE SURFACE B-CELL RECEPTOR LIGHT CHAIN IN CASES OF HAIRY CELL LEUKEMIA OCCURRING AT EXTRAFOLLICULAR SITES

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Ig gene analysis delineates critical features of the clonal history of a B-cell tumor. After antigen interaction, mature B-cells undergo somatic mutation of the V-genes and isotype switch events, generally in the germinal center (GC). Receptor revision by secondary recombination of the V-genes with re-expression of recombination activating gene (RAG) enzymes rarely occurs at this stage. From small series of cases, we have reported that most hairy cell leukemias (HCL) carry mutated VH-genes, with low levels of intraclonal heterogeneity, while a minor subset have unmutated VH-genes. Both subsets commonly have ongoing Ig isotype switch events and express activation-induced cytidine-deaminase (AID). However they lack CD27 and CD38 GC markers, and CD23, essential for lymph node entry. In an expanded series of HCL (60 cases) with VH-genes available, the expressed VL (32) tumor-derived genes were evaluated to probe more fully the differentiation status of the cell of origin. The majority (35/44, 79,5%) co-expressed multiple Ig isotype proteins on the HCs. From analysis of VH, VH3 family was most common (38/60, 63%), with significant preference of the VH3-30 and VH3-33 members (p<0,005). Most HCL (44/60) carried variable tiers of mutations in the VH-genes (77.13-97.95% homology to germline), with low level of intraclonal heterogeneity also documented in cases (13/60) with <2% deviation from germline, while 3/60 (5%) displayed completely unmutated VH-genes. Analysis of the light chains showed preferential use of surface lambda chain (34/50, 62%), consistent with secondary rearrangement. VL-genes were evaluated in 16 kappa and 16 lambda expressing HCL. All (16/16) lambda cases used Jl3 segment. Thirty of 32 cases carried mutated VL-genes (94,75%-99.6%) with low levels of intraclonal heterogeneity, while 2 cases carried completely unmutated VL-genes, reflecting heterogeneity in mutational status as for the VH-gene. Strikingly, cloning of the tumor VL revealed in-frame functional secondary rearrangements in 2/13 cases (Vk1 & Vk2 in Case R1, Vl1 & Vl2 in R2), most likely occurring in different tumor cells. Primary and secondary rearrangements showed mutations (98.1 and 99.6% homology in R1; 97.6 and 99.6% homology in R2). In both cases, RAG1 re-induction was also identified by RT-PCR and sequence verification. Both cases expressed AID transcripts, displayed intraclonal mutational variation in the VH and/or the VL-genes and 1/2 cases had ongoing isotype switch events. These data suggest a dynamic, on-going modification of the B-cell receptor (BCR) in HCs, including receptor revision, which occurs most likely in response to antigenic stimuli. N-glycosylation sites, commonly introduced by somatic mutation in the BCR of tumors of the GC, were not observed in the functional VH or VL-genes, to support the concept that tumor events occur outside the GC. These data confirm heterogeneity in the cell of origin in terms of mutational status, with a minor subset with unmutated V-genes. Restricted V-gene segment usage, and low levels of ongoing mutations with AID activated, coupled with the new observation of receptor revision and re-expression of RAG enzymes indicate that selective BCR stimulation could be a promoting factor in HCL development at extrafollicular sites.

PO-073

CD38 AND ZAP-70, TWO INDEPENDENT NEGATIVE PROGNOSTIC MARKERS IN CHRONIC LYMPHOCYTIC LEUKEMIA, ARE FUNCTIONALLY LINKED

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CD38+ chronic lymphocytic leukemia (CLL) patients, a significant subset of all CLL cases, are characterized by earlier resistance to treat-

ment and poor survival. Beside CD38 expression, the molecular signature of aggressive CLL includes absence of mutations in the IgVH genes and presence of ZAP-70. We have previously shown that CD38 is directly involved in the delivery of potent proliferation/survival signals to CLL cells mainly through the binding of its counter-receptor CD31, expressed by a population of nurse-like cells. Follow-up of those data shows the existence of a functional link between CD38 and ZAP-70. This conclusion was inferred after studying CD38-mediated signals in 15 molecularly characterized (Ig mutational status, CD38 and ZAP-70 expression and chromosomal abnormalities) CLL patients. Tyrosine phosphorylation of ERK1/2 was selected as an indicator of the presence of a CD38 signaling pathway, dividing the sample in responders and non-responders. The single parameter significantly different between the two groups (p<0.048) was the presence of ZAP-70 >10% within the cytoplasm of the responders. Futher, ZAP-70 becomes tyrosine phosphorylated upon CD38 ligation in a B cell line model transfected with a myc-tagged ZAP-70 and used as a playground to set up optimal experimental conditions and to select highly specific reagents. This finding could be also confirmed in a subset of 4 CLL patients. A final confirm of the functional relevance of ZAP-70 for CD38-mediated signals comes from the observation that IL-2 (previously shown to induce surface CD38 expression and to increase the signaling potential of the receptor) also increases the expression of cytoplasmic ZAP-70, as seen in flow cytometry and in western blot in 4 out of 6 tested samples. Together, these results provide the first evidence of a functional link between two previously unrelated negative prognostic markers in CLL patients.

PO-074

CONSOLIDATION AND MAINTENANCE THERAPY WITH RITUXIMAB IMPROVES CLINICAL OUTCOME WITHIN ZAP-70 POSITIVE CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) PATIENTS

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Clinical trials of monoclonal antibodies in combination with chemotherapy have reported improved outcome in CLL because this approach reduces disease burden to minimal levels detectable only by flow cytometry or molecular methods. Along this line, we have recently published that rituximab in sequential combination with fludarabine (Flu) for symptomatic, untreated CLL allowed us to achieve higher remission rates and a longer duration of response (Del Poeta et al, Cancer, 2005). Recent literature data indicate that unmutated VH genes, CD38 and/or ZAP-70 protein tyrosine kinase overexpression may predict a worse outcome. We performed a phase II study that added rituximab sequentially to Flu as initial therapy for symptomatic, untreated CLL in order to evaluate both the clinical response and outcome. ZAP-70 protein was determined before chemotherapy on mononuclear cells by flow cytometry using anti-ZAP-70 Alexa Fluor 488 (Caltag Laboratories) conjugated antibody. Seventy-two CLL patients, median age 60 years (range 37-74) received six monthly courses of Flu (25 mg/sqm for 5 days) and four weekly doses of rituximab (375 mg/sqm) starting on an average of thirty days (range 21-150) after completion of Flu therapy. According to modified Rai stages, 4 patients had a low stage, 67 an intermediate stage and 1 a high stage. Based on NCI criteria, 56/70 (80%) patients achieved a complete remission (CR), 12/70 (17%) a partial remission (PR) and 2/70 (3%) a stable disease (SD). Three patients presented grade 3 (WHO) infective lung toxicity and 1 patient acute fatal B hepatitis. Hematologic toxicity included mainly neutropenia (grade 3 and/or 4 in 37 pts) and thrombocytopenia (grade 3 and/or 4 in 4 pts). Twenty eight patients, either with CD5+CD19+ bone marrow cells >1% (n=17 pts) or presenting CD19+CD5+ peripheral lymphocytes >1000/microl (n=11 pts) within six months after completion of the induction treatment, underwent consolidation/maintenance therapy with four monthly cycles of rituximab at 375 mg/sqm followed by eight/twelve monthly doses of rituximab at 180 mg/sqm. The median follow-up duration was 36 months. Noteworthy, all B-CLL pts experienced a very long progression-free survival (PFS) from treatment (71% at 5 years). Nevertheless, CLL patients that underwent consolidation therapy showed a significant longer duration of response (87% vs 54% at 5 years, p=0.02). ZAP-70 was positive (>20%) in 35/72 (49%) pts and a significant shorter PFS was observed in ZAP-70+ pts (36% vs 95% at 5 years; p=0.0002). Noteworthy, within the consolidated patients subset (n=28), ZAP-70+ pts (n=12) showed a worse PFS (68% vs 100% at 5 years, p=0.02). However, interestingly, within the ZAP-70+ subset (n=35), the consolidated patients (n=12) showed a significant longer duration of response (68% vs 0% at 2.6 years, p=0.01) in comparison with the unconsolidated patients (n=23). Therefore, the addition of consolidation/maintenance therapy with rituximab prolongs significantly duration of response allowing a better outcome. Finally, this immunotherapeutic supplement seems to improve significantly the clinical outcome of patients notoriously at bad prognosis, such as ZAP-70+ B-CLL.

PO-075

ZAP-70 EXPRESSION AS A PREDICTOR OF DISEASE PROGRESSION IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA.

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The clinical heterogeneity that characterizes chronic lymphocytic leukemia (CLL) poses critical questions concerning the identification of high risk patients. Unmutated IgVH genes, CD38 and ZAP70 expression >20% cut-off value have emerged in recent years as the most useful tools in identifying aggressive CLL. The aim of this work is to evaluate the power of ZAP-70 expression in predicting disease progression. To do so, we evaluated ZAP-70 expression in 121 patients with CLL (74 M; 47 F; mean age 63 yrs; range 35-88 yrs), following the method described by Crespo *et al*. (NEJM, 2003) and adopting a ≥ 20% positivity threshold. Fourty-nine patients (40%) were ZAP-70+. No significant difference in age, sex, Binet clinical stage, hemoglobin levels, and Kappa or Lambda light chain restriction at diagnosis were found between ZAP-70+ and ZAP-70- CLL patients.

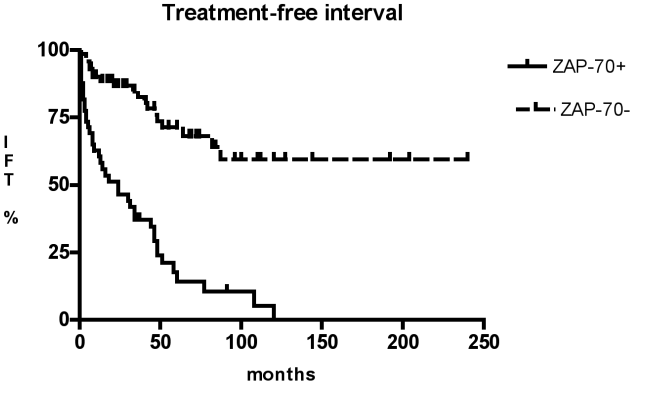

Figure.

However, ZAP-70 positivity strongly correlated with IgVH unmutated status (p<0.0001), CD38 expression (p 0.02), higher level of peripheral blood lymphocytosis (p 0.05), and need for treatment (p<0.0001). Further, ZAP-70+ patients displayed a significantly shorter treatment-free interval [26.6 months vs 53.8 months, (p <0.0001)], as shown in Figure 1. In conclusion, our data show that ZAP-70 is a reliable prognostic marker in B-CLL, suggesting that expression of the molecule should become an integral component of the CLL diagnostic grid. However, further studies are needed to standardize the most reproducible flow cytometric protocol.

PO-076

SERUM LEVELS OF TH1/TH2 CYTOKINES IN B-CLL PATIENTS

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Chronic lymphocytic leukemia (CLL) B cells are believed to display prolonged survival as a result of defective apoptosis. However, CLL B cells spontaneously undergo apoptosis *in vitro*, which suggests a role for the *in vivo* microenvironment in keeping the cell death program inhibited in clonal B cells. Moreover, previous studies have shown the ability of sera from B-CLL patients to prolong survival of clonal B cells *in vitro*. In an attempt to identify such survival factors for CLL B cells, we measured the serum concentrations of nine different Th1 and Th2 cytokines (namely, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, IFN-γ, TNF-α) in 30 consecutive B-CLL patients in different Rai stages as well as in 10 healthy subjects. Serum cytokine concentrations were quantified using a proteomic ELISA assay (SearchLight (TM) Human Th1/Th2 Cytokine Array, Pierce Endogen, Rockford, IL, USA), according to the manufacturer's instructions. Means ± standard deviations for serum cytokine concentrations, expressed in pg/mL, were as follows: IL-2, 3.2±3.6; IL-4, 1.8±3.7; IL-5, 12.9±15.8; IL-8, 4.9±6.1; IL-10, 1.6±4.8; IL-12, 6.7±9.0; IL-13, 57.0±152.0; IFN-γ, 2.0±3.1; TNF-α, 16.4±41.2. Levels of Th1/Th2 cytokines showed a wide range of variability across B-CLL subjects and appeared to correlate neither with Rai stage nor degree of lymphocytosis; when compared to normal subjects, only IL-8 serum levels were shown to be consistently more elevated in B-CLL patients, although no correlation could be found again with clinical stage or laboratory parameters. To substantiate the role for elevated IL-8 levels in B-CLL patients' sera, we plan to measure serum Th1/Th2 cytokine concentrations in at least 30 more patients in different Rai stages as well as to assess whether addition or inhibition of IL-8 in CLL B cell cultures may have an impact on *in vitro* survival of clonal B cells.

PO-077

LOW DOSE CAMPATH 1-H IS HIGHLY EFFECTIVE AND SAFE IN ADVANCED CHRONIC CLL

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Introduction. Campath 1H has already shown its efficacy both in refractory and untreated CLL. Moreover, this monoclonal antibody can ameliorate the quality of response in patients pre-treated with fludarabine-based chemotherapy. The severity of Campath-related adverse events is markedly reduced by the subcutaneous administration. We present an update of the results obtained with a prolonged treatment with low dose subcutaneous Campath in refractory CLL. (Cortelezzi A. *et al.* Haematologica 2005, 90: 410-412). Since January 2003 we treated at our Istitutions thirty-five consecutive CLL patients. Patient characteristics were as follows: M/F 22/13; median age 68 years, range 48-83; Binet stage C 34.3%, stage B 57.1%, progressive stage A 8.6%; abnormal karyotype 74.3%, including unfavourable alterations (17p- 17.1%, 11q- 25.7%, trisomy 12 17.1% and 6q- 2.8%); ZAP-70 positive at immunoistochemistry 60%. All the patients were pretrated (median prior lines of therapy 2, range 1-5) and refractory to alkylators, 57.1% were also fludarabine-resistant, and 22.8% rituximab-resistant. At least one negative prognostic factor (including Binet's stage C, CD38 or ZAP70 positivity, 17p-, 11q- chromosomal alterations, complex kariotype, and fludarabine refractoriness) was evidenced in 88.6% of the patients. *Treatment.* Alemtuzumab was given subcutaneously three times a week at 10 mg per dose, after an initial dose of 3 mg on day 1. Treatment was given for 18 weeks, for a cumulative dose of 543 mg; it was prematurely stopped in the case of progressive disease on therapy, patient consent withdrawal, or if a complete response was achieved before the completion of the planned therapy period. Treatment was temporarily withdrawn if the peripheral blood neutrophil count fell to less than 0.5×10^9/L, notwithstanding supportive G-CSF therapy, if the platelet count fell to less than 20×10^9/L, or if there were severe infectious complications. Patients received paracetamol (1 g orally) and chlorphenamine (10 mg intravenously) prior to each injection until injection-related side effects had subsided. In the case of local WHO grade β 2 side effects, hydrocortisone 5 mg was added to the alemtuzumab-containing syringe until skin reactions subsided. All patients received anti-infective prophylaxis with trimethoprim and sulfamethoxazole (double strength; twice daily, three times a week) and acyclovir (400 mg t.i.d.) during alemtuzumab therapy and for eight weeks after its completion.

Group	OR	CR	PR	SD	PD
All patients (n=35)	51.4	25.7	25.7	34.3	14.3
Failed fludarabine (n=20)	50.0	25	25	35	15
Failed rituximab (n=8)	37.5	25.0	12.5	50	12.5
Stage C (n=12)	50	25	25	33.3	16.6
Age ≥ 65 years (n=27)	48.1	22.2	25.9	37.1	14.8
Unfavourable cytogenetic(n=23)	43.6	21.8	21.8	31.4	5.7
17p- (n=6)	66.6	33.3	33.3	33.3	0

Figure. Response (NCI criteria, %) to low-dose subcutaneous alemtuzumab in 35 refractory B-CLL patients.

Results. The overall response rate (NCIWG criteria) was 51.4%, including 25.7% complete response. Better responses were seen in blood (77.2%), as compared with bone marrow (55.5%), spleen (57.9%), and lymph nodes (51.4%). Responses were observed in patients with Zap70+ (42.8%), adverse karyotype (43.6%), 17p- (66.6%), stage C (50%), fludarabine- (50%) and rituximab-refractoriness (37.53%), as well as in patients with age > 65 (48.1%) and > 70 (46.1%). Progression was documented in 8/18 responders after a median of 12 months (range 6-35 months). After a median follow-up of 16 months (range 1.5-40) the median survival has not been reached for the entire case series (65.7% alive), responders (77.7%), and non-responders (52.9%). Twelve patients (8 non-responders and 4 responders to Campath therapy) died after a median of 12.5 months (range 1.5-25); two of them were in still in remission and 10 in progressive disease. Infectious complications were the direct cause of death in 10/12 patients. Grade III-IV neutropenia or thrombocytopenia were recorded in 34.3% and 2.8% of the patients, respectively. Mild anaemia was observed in 51.4% of the patients during alemtuzumab therapy. Coombs-positive AIHA was documented in two patients after the end of Tx administration (9 and 1.5 months), being a reactivation in one of them. Two patients developed ITP one month after stopping alemtuzumab. Infections (1 otitis media due to Pseudomonas, 2 dermatomeric Herpes zoster, 2 pneumonia, 1 lethal polymicrobial sepsis, 4 FUO, 1 UTI, 1 oropharingeal candidiasis) occurred during alemtuzumab treatment in 34.3% of the patients. During the entire follow-up infections were evidenced in 20 patients (57,1% of our casistics). A total of 32 infectious episodes were recorded: 17 pneumonias (including 2 PCPs and 2 pulmonary aspergillosis), 6 polymicrobial sepsis, 1 perianal Pseudomonas abscess, 2 dermatomeric Herpes zoster, and 6 FUO. Transient and clinically silent reactivation of cytomegalovirus was documented in 22.8% of the patients by pp65 antigenemia testing or PCR. All patients was treated with oral ganciclovir for two weaks with rapid negativization of CMV viremia. Eight patients (4 cases with latent and 4 with reactivated HBV infection) received lamivudine while on alemtuzumab. Adefovir dipivoxil was associated to lamivudine in two cases for an hepatitis flare. These antiviral therapies enables us to complete alemtuzumab Tx in all the patients, despite CMV or HBV reactivation. *Conclusion.* We confirm on a larger number of high-risk relapsing/refractory CLL patients with a longer median follow-up, the high percentage of response, long remission duration, and the favourable toxicity profile of ld sc alemtuzumab, already shown in the pilot study.

PO-078

DIFFERENTIAL DIAGNOSIS BETWEEN SÉZARY SYNDROME AND T-CELL PROLYMPHOCY

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Cytomorpho-immunophenotypical criteria and the demonstration of a T-cell clone in the skin and in the peripheral blood (PB) are essential for the diagnosis of Sézary Syndrome (SS), in addition to erytrhoderma and lymphadenopathies. T-cell prolymphocytic leukaemia (T-PLL) is a rare, aggressive, post-thymic T-cell malignancy accompanied by progressive lymphocytosis, splenomegaly, often skin lesions. Laboratory and clinical data suggest great similarity between SS and T-PLL. We describe a case of T-PLL mimicking a SS. In March 2004 a white man of 63 years developed a rapidly growing and slightly itching squamous macular skin lesion on the interscapular region: histopathology and immunohistochemistry were consistent with Mycosis Fungoides (MF): superficial dermal band-like infiltrates of small CD3+ CD4+ CD8- lymphocytes with epidermotropism, some Pautrier's microabscesses. In PB there were CD4, CD3, CD2, CD5, CD7 positive T lymphocytosis (14000/mmc) with T cell receptor (TCR) Vβ5.1 chain monoclonality. Serology for HTLV-I and II was negative. Bone marrow (BM) biopsy demonstrated an interstitial CD3+ CD5+ linfoid infiltrate; total body CT scan resulted negative. TCRγ and δ gene rearrangement patterns in PB and BM showed, respectively, a monoclonal Jpγ and an oligoclonal Vδ3Jδ1 rearrangement. Electronmicroscopy revealed lymphocytes with cerebriform nuclei and deep basophilic cytoplasm resembling prolymphocytes of T-PLL or Sézary cells. Cytogenetic analysis using FISH techniques revealed add7q, i8q, 10p+, 12p-. In September the patient started fludarabine 25 mg/m^2 (days 1 to 5 every 4 weeks) for 5 courses. In April 2005 he didn't show skin lesions, CT scan was negative, but lymphocytosis increased (45000/mmc); the BM biopsy and the cytogenetic analysis of PB confirmed the above mentioned alterations. Cytofluorimetric analysis of the CD4+ CD5+ CD7+ population showed two different pattern as regarding CD3 positivity; half of the T cells was expressing CD3 antigens on the membrane surface, the other half in the citosol; both isoforms of the molecule expressed the same Vβ5.1 chain; besides there was an elevated fluorescence intensity of CD5 and CD7, characteristic of T-PLL. The study of TCRγ and δ gene rearrangement in PB showed, respectively, a monoclonal Jpγ and a monoclonal Vδ3Jδ1 rearrangement. In May 2005 the patient started therapy with alemtuzumab 30 mg subcutaneously 3 times a week. At the end of the 5th week a complete haematological, immunophenotypical and cytogenetic response was obtained, even if the molecular analysis showed a persistent clonal expansion of T cells. Three months later the patient had progression of disease with lymphocytosis (56000/mmc), massive bone marrow infiltration, multiple superficial and visceral lymphoadenopathies, lung and hepatic nodules, spleen involvement. Salvage therapy with DHAP schedule failed and the patient died. This case underlines that the currently proposed haematological, flowcytometric and molecular criteria for SS are not forceful enough to differentiate between SS, T-PLL and some MF with blood involvement. As regarding a more detailed characterization of Sézary cells some investigators suggest that a loss of T-cell lineage markers together with the amount of CD4+ CD26- cells in PB, in an appropriate clinical and histological contest, could be a very sensitive criterion of SS.

PO-079

ORAL FLUDARABINE AND CYCLOPHOSPHAMIDE IN UNTREATED PATIENTS AFFECTED BY CHRONIC LYMPHOCYTIC LEUKEMIA. A PILOT STUDY.

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The introduction of fludarabine into the treatment of chronic lymphocytic leukemia (CLL) has improved the complete remission rate (CR), overall response rate (OR), and progression free survival (PFS) compared with alkylator based regimens. Its synergistic action with cyclophosphamide has demonstrated significative advantages as front line therapy in untreated CLL patients with advanced disease. The oral formulation of fludarabine showed a similar safety profile and rensponse rate as the endovenous compound. Primary end-point was to test efficacy and safety of the oral formulation of fludarabine combined with cyclophosphamide as front-line therapy of high-risk CLL. As secondary end-point we examined the impact of new prognostic factors associated with progressive CLL (i.e. unmutated IgVH gene status, positivity for ZAP-70, del(11)(q23), and del(17)(13.1)) on treatment outcome. Starting from December 2002, 31 untreated patients with advanced CLL, 21 male and 10 female, with a median age of 68,5 years (52-75) received oral fludarabine (30 mg/sm) and oral cyclophosphamide (250 mg/sm) for 3 consecutive days every 4 weeks, for a total of 6 cycles. At study entry, 26 patients were in stage B/II with progressive disease, 2 in stage C/III, and 3 in stage C/IV. Thirteen patients had unmutated and 11 mutated IgVH genes, while in the remaining 7 patients the IgVH gene mutation status was not evaluable. Fifteen patients had more than 20% ZAP-70 positive CLL-B cells, and five patients had the high rish cytogenetic abnormalities del(11)(q23) or del(17)(13.1). Among the 29 evaluable patients, 15 obtained CR (52%), 6 PR (21%). Of the remaining patients 5 had stable disease and 3 progressive disease. In terms of haematological toxicity 6 patients developed grade IV neutropenia and received G-CSF treatment, while two patients developed severe anemia (grade III and IV) that required red blood cell transfusions. Only one patient developed a transient febrile neutropenia of unknown origin, but did not require hospitalization. Mild extra-hematological toxicity consisting of nausea and vomiting occurred in six patients during the treatment. No significant differences were noticed in terms of CR and OR rate between the IgVH mutated and unmutated groups (p=ns). Among the 6 patients who have relapsed so far, 4 had unmutated and only 1 had mutated IgVH genes (p=ns), and among the 4 patients that required new treatment (NCI WG criteria), 3 had unmutated IgVH. Oral fludarabine plus cyclophosphamide as front-line therapy in CLL achieved a good overall response rate in our series of patients. The haematological and extrahematological side effect were mild and the oral scheme was easy to administer. The differences in terms of CR, OR and PFS between the IgVH mutated and unmutated groups did non reach statistical significance. However, a longer follow-up is required to define the possible correlation between these prognostic factors and treatment outcome.

PO-080

LOW-DOSE THALIDOMIDE IN COMBINATION WITH ORAL FLUDARABINE AND CYCLOPHOSPHAMIDE IS INEFFECTIVE IN HEAVILY PRETREATED PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Elevated levels of TNF-α have been associated with progressive disease and shorter survival in patients with chronic lymphocytic leukemia (CLL). Thalidomide is a potent immunomodulating, antiangiogenetic and antitumor agent that has been shown to inhibit production of TNF- α. *Objective.* We therefore investigated the effects of thalidomide on clinical outcome and TNF- α; serum levels in five previously treated patients with progressive CLL. *Design and Methods.* The treatment schedule consisted of daily Thalidomide, 100 mg from day 1 to 180, combined with oral Fludarabine (30 mg/m^2) and oral Cyclophosphamide (250 mg/m^2) that were given for three consecutive days and repeated every 28 days for six cycles, starting from day +10 of Thalidomide administration. *Results.* Median duration of treatment was 60 days (range 30-120). Treatment was discontinued in four patients because of disease progression and in one patient because of mild neurological toxicity. Serum TNF-α levels did not show any decrease during treatment. Median serum TNF-α level in the control group of healthy donors was 9.8 pg/mL (range 4.4-12.3 pg/mL), whereas pre-treatment median serum TNF-α level in the CLL patients was 15.1 pg/mL (range 10-16.6 pg/mL). The TNF-α levels gradually increased in all patients during therapy, with a median value of 15.5 pg/mL (range 11.3-31.4 pg/mL) and 19.5 pg/mL (range 10.8-51.7 pg/mL) on days 10 and 30, respectively. On the last day of treatment all patients had higher serum TNF- α levels than before treatment. *Interpretation and Conclusion.* Low dose Thalidomide is not effective in CLL patients with refractory disease. However, considering that the treatment was relatively well tolerated and safe, higher Thalidomide doses may be worth further investigation.

PO-081

ANTIBODY-MEDIATED APOPTOSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA B CELLS: PRELIMINARY DATA

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B-cell chronic lymphocytic leukemia (B-CLL), the most frequently diagnosed form of leukemia in adults in the Western hemisphere, is characterized by a progressive accumulation of long-lived and well-differentiated clonal B lymphocytes in the peripheral blood, bone marrow, and lymphoid tissue. Although the pathogenesis of B-CLL is not fully elucidated, the progressive increase in lymphocyte counts coupled with the very low proportion of proliferating cells has led to the notion that B-CLL may be primarily determined by defective apoptosis. Very recently, our group set out to characterize the effects of a monoclonal antibody capable of reducing survival in CLL B cells. In *in vitro* time-course experiments, survival was reduced by at least 30% (range, 30-50%) in CLL B cells cultured in the presence of the monoclonal antibody for six days with respect to control cultures, as assessed by the MTT test and cytofluorometric analysis (six independent experiments thus far). This antibody appears to recognize an antigen widely expressed (range, 46-66%) by and restricted to normal and CLL B cells, which is distinct from CD20, as shown by competition experiments. Studies are ongoing to better characterize the mechanisms by which the antibody can determine reduced survival of CLL B cells and to identify the surface antigen triggering these effects.

PO-082

MARKED REDUCTION OF PERIPHERAL BLOOD LYMPHOCYTOSIS AFTER LAMIVUDINE THERAPY: A POSSIBLE, UNEXPECTED RELATIONSHIP BETWEEN HEPATITIS B VIRUS (HBV) AND B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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A 81 year-old male presented at our Institution on March, 2006, because of marked peripheral blood leucocytosis (200.000/uL), fever and worsening of general conditions. At physical examination, he showed moderate splenomegaly (4 cm below costal margin), but no significant superficial lymphnodes. Hb was 12.5 g/dL, platelet count 456.000/uL. Morphological analysis of both peripheral blood and marrow cellularity evidenced the almost exclusive presence of a homogeneous population of small-medium sized lymphocytes with clumped nuclear chromatin, expressing a clonal immunephenotype (CD19+, CD20+, CD23+, CD5+, CD38-, ZAP-70-, CD10 -, FMC7-, restricted expression of surface lambda light chains immunoglobulins at low density). Bone histology confirmed a diffuse marrow infiltration by a low grade lymphoproliferative disorder. LDH was 691 U/L, serum creatinine 1.8 mg/dL. Electrophoresis of serum proteins revealed a moderate hypo-gammaglobulinemia, while liver enzymes, clot tests, uric acid and karyotype were normal. Ultrasound analysis did not evidence enlarged abdominal lymphnodes, X-ray showed possible lung fibrosis. After hydration, peripheral lymphocytosis was confirmed, while serum creatinine returned to normal values. Antibodies against hepatitis C virus (HCV) were not detected, but the patient expressed some markers of hepatitis B virus (HBV) infection (HBsAg+, total HbcAb+ and HBeAg+), whereas HbsAb, HbcAb IgM and HbeAb were negative. HBV-DNA copies at PCR were > 60×10^6/mL. The patient was not aware of his HBV infection. A diagnosis of symptomatic, typical B-cell chronic lymphocytic leukaemia (B-CLL) in an *active* HBV carrier was made. According to current guidelines, the patient started a prophylactic treatment for HBV with lamivudine (100 mg/d), delaying the planned chemotherapy. However, when he returned for the first control two weeks later, WBC count was significantly reduced (26.700/uL) and this phenomenon was confirmed after further 2 weeks of lamivudine. LDH levels also had decreased to normal values. At this point, we decided to continue with antiviral therapy, avoiding of starting any chemotherapy treatment. After 8 weeks of lamivudine therapy, the patient maintains a moderate peripheral lymphocytosis (WBC 20.500/ul), with a significant improvement of his general condition and a marked reduction of viral load (HBV-DNA copies 1.8×10^6/mL). A clear relationship between HCV infection and at least some subtypes of lymphoproliferative disorders has been demonstrated by numerous epidemiological, clinical and biological evidences. Of interest, antiviral therapy with interferon ± ribavirin is effective in inducing clinical remissions in some low-grade lymphoproliferative disorders with concomitant HCV infection. The same correlation has not been clearly shown in patients with HBV infection, but very recent data support the hypothesis that also HBV could be associated with lymphoid neoplasms, although to a lesser extent than HCV (Marcucci *et al.*, Haematologica 2006). In our patient, we observed a clear reduction of tumor mass after an effective antiviral treatment with lamivudine, without the adjunct of other cytostatics. Although we have no definitive elements to demonstrate that HBV was involved in determining or facilitating progression of B-CLL, this case report suggests the possibility of a relationship between HBV infection and the lymphoproliferative disorder developed in our patient.

Acute Myeloid Leukemia I

PO-083

MOLECULAR CYTOGENETIC ANALYSIS OF ACUTE MYELOID LEUKEMIA PATIENTS: A SINGLE CENTRE EXPERIENCE OF DIAGNOSTIC FLUORESCENCE *IN SITU* HYBRIDIZATION FOR THE DETECTION OF GENOMIC ABNORMALITIES

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Background and Objectives. Karyotype is one of the most important prognostic factors in acute myeloid leukemia (AML). From previous studies, FISH does not seem to improve cytogenetic risk assessment in patients with adequate pretreatment karyotype information, but can add important informations in patients with normal karyotype or without analyzable methaphases. *Design and Methods.* In a ten years period (1996-2006) 131 consecutive patients were diagnosed with AML (M0=2, M1=21, M2=38, M3=4, M4=50, M5=15, M6=1) at the Hematology Division of University of Siena. Median age was 66 (range 11-90). Conventional cytogenetic analysis evidenced a karyotypic abnormality in 71 pts (68%) of the samples, while 60 patients (42%) had a normal karyotype or not analyzable metaphases. Eleven patients had a favorable karyotype (i.e.t(15;17), t(8;21), inv(16); 84 patients had an intermediate karyotype (+8, normal karyotype); 30 pts had an unfavorable karyotype (3 abnormalities, MLL abnormalities, -5,-7). To assess the diagnostic value of molecular cytogenetics in AML patients, we used fluorescence in situ hybridization (FISH) applying a comprehensive DNA-probe set, i.e. -5q, -7q, +11q, t(15;17), t(8;21),+13, +21, for the detection of some of the most relevant AML-associated chromosome aberrations. We studied in a retrospective series 55 (40%) patients with a normal karyotype. *Results.* FISH identified 1 case of MLL amplification; 1 case with inv(16);1 case of +13; 1 case of +21; 1 case of del 5q; 3 cases of PML/RARa rearrangement not identified because of poor methaphase morphology. Molecular cytogenetic analysis was also more sensitive for the detection of genomic imbalances, in particular +11q, +21, PML/RARa, in patients with normal karyotypes but poor chromosome morphology, in patients with a leukemia-specific balanced rearrangement, or in patients with complex karyotypes. *Conclusions.* Our results support the use of FISH as a complementary method for the detection of abnormalities in AML patients eligible for intensive therapy. Molecular cytogenetics should also be considered in cases with insufficient yields of metaphase cells, poor chromosome morphology, or both.

PO-084

ACUTE MYELOID LEUKEMIA WITH T(Y;1)(Q12;Q23) IN A CHILD WITH KLINEFELTER SYNDROME

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Background. Different molecular events underlie the t(Y;1)(q12;q21-23) translocation which has been described in myeloid and lymphoid malignancies. Using fluorescence *in situ* hybridization (FISH) 1q breakpoints were narrowed telomeric to the heterochromatic region in one case of myelodysplastic syndrome and in one case of acute myeloid leukemia (AML), and within the centromeric region in one patient with acute lymphoblastic leukemia. *Aim.* Molecular characterization of t(Y;1)(q12;q23) in a child with AML. *Materials and Methods.* An 18-month old infant developed M0 subtype AML. Blasts were myeloperoxidase negative and showed the following positive antigens: CD13, CD33, HLA-DR, CD4, CD11b, CD7. Bone marrow karyotype was 47,XX,+der(Y)t(Y;1)(q12;q23)[15]. Constitutional karyotype (47,XXY) revealed a Klinefelter syndrome. We used double colour metaphase FISH with a centromeric probe for chromosome 1 heterochromatin and α satellite sequences (Vysis, Olympus Italia), a panel of DNA clones for 1q21-q25, and six clones for Yq11-q12. Analyses were performed in 8-10 abnormal metaphases with a fluorescence microscope (Provis, Olympus, Milan, Italy) equipped with a cooled CCD camera (Sensys, Photometrics) run by PathVysion software (Vysis, Stuttgart, Germany). *Results.* FISH showed chromosome Yq12 breakpoint fell either within clone RP11-242E13 or between clone RP11-242E13 and clone RP11-57J19. The 1q breakpoint was narrowed at band q23 between two contiguous clones, i.e RP11-192B4 (centromerically) and RP11-195C7 (telomerically). *Conclusions.* Chromosome Yq12 breakpoint contains abundant heterochromatic sequences but no known genes. Chromosome 1q23 breakpoint fell within the RABGAP1L gene (RAB GTPase activating protein 1-like) which encodes a 298 amino acid protein with a putative phosphotyrosine binding domain. In mice, RABGAP1L is strongly expressed in both erythroid and megakaryocyte lineages, and in multipotential CFU-GEMM colonies suggesting it may play a regulatory function also in human hematopoiesis. Notably, transcriptional gene expression studies in human esophageal squamous cell carcinoma detected RABGAP1L up-regulation in cases with nodal localization. We report for the first time involvement of RABGAP1L in hematological malignancies.

Fondazione Cassa di Risparmio di Perugia, PRIN-MIUR (Ministero per l'Istruzione, Università e Ricerca scientifica) Italy.

PO-085

ACUTE MYELOID LEUKEMIA-DERIVED DENDRITIC CELLS EXPRESS THE IMMUNOREGULATORY ENZYME INDOLEAMINE 2,3-DIOXYGENASE

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Acute myeloid leukemia (AML) cells may be differentiated into dendritic cells (DC) which have increased immunogenicity, but retain some immunosuppressive features of leukemic cells. Indoleamine 2,3-dioxygenase (IDO) enzyme, which catalyzes the conversion of tryptophan into kynurenine, has been identified as a novel immunosuppressive agent by inhibiting T-cell proliferation and is involved in tolerance induction to tumors. We have recently shown that IDO protein is constitutively expressed in a significant subset of newly diagnosed AML patients, resulting in tryptophan catabolism along the kynurenine pathway and in the inhibition of allogeneic T-cell proliferation. We, then, *in vitro* generated DCs from 7 AML samples (AML-DCs) in the presence of GM-CSF, IL-4 and TNF-α. The cells we obtained were morphologically and phenotypically semi-mature DCs expressing CD40, CD80, CD86, HLA-DR and CD1a molecules and they were more efficient to induce T-cell proliferation and type 1 cytokine production than primary AML blasts. At baseline, 5/7 AML samples expressed IDO, whereas 2/7 did not. After differentiation into DCs, IDO+ AML samples showed an up-regulation of IDO mRNA and protein, and IDO- AML cells turned positive. IDO-expressing AML-DCs were capable to catabolize tryptophan into kynurenine metabolite and, functionally, they inhibited allogeneic T-cell proliferation through an IDO-dependent mechanism. These data identify IDO-mediated catabolism as a tolerogenic mechanism in AML-DCs and have clinical implications for the use of AML-DCs as cellular vaccine against leukemia.

PO-086

SINGLE-AGENT SU11657, A NOVEL FLT3 INHIBITOR, SHOWS BIOLOGIC ACTIVITY IN ACUTE MYELOID LEUKEMIA CELLS *IN VITRO*

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fms-related tyrosine kinase3 (FLT3) is one of the most commonly mutated gene in human acute myeloid leukemia (AML) and has implicated in its pathogenesis. Constitutive activation of the FLT3 receptor tyrosine kinase, has been linked either by internal tandem duplication (ITD) of the juxtamembrane region or by point mutation in the second tyrosine kinase domain (TKD). The purpose of the study was to evaluate, *in vitro*, the effect and the biological activity of SU11657 (Pfizer), a

new compound FLT3 kinase inhibitor. SU11657 was investigated on human cell lines from AML patients (MV4-11 and HL-60) and blast from patients AML using a wide range of concentrations (1nanomolar-10micromolar). FLT3 expression levels were evaluated by flow cytometry. Furthermore, to evaluate the effect of SU11657 we analyzed the cytotoxicity, induction of apoptosis and inhibition of cell proliferation by flow cytometry. The antiproliferative and cytostatic effects of SU11657 were confirmed by analysis of signal transduction. HL-60 cell line served as a control as it expresses a wild type receptor. MV4-11 is a cell line that expresses a naturally internal tandem duplication (-ITD) in homozygous form. In HL-60 does not show relevant effect after treatment with SU11657. Instead, in MV4-11 we observed a decrease dose-dependent in cell viability after treatment with SU11657. The effects of this compound on cell cycle progression show an accumulation of G1/S phase and an induction of apoptosis at 1-10nanomolar concentration after 24h of treatment. First we observed a dephosphorylation of FLT3 on Tyr(591) in whole cell extracts from MV4-11 cells after treatment with SU11657 100nanomolar. We also demonstrated a hypophosphorylation of AKT on Ser(473) and a consequently dephosphorylation of BAD on Ser(136) at nanomolar concentration. We observed a dephosphorylation of STAT-5 to 100nanomolar of Su11657 at 24h. We evaluated the effects of this new compound in AML primary progenitors that showed FLT3-ITD, FLT3-TKD and FLT3-wt. In the patients with mutation ITD and TKD was evident a modification of cell cycle progression with a decrease in G2/M phase and an increase of subdiploid peak. The effect of SU11657 in patients FLT3-wt was not relevant. Due to its FLT3 inhibitory activity, SU11657 represent promising compound for clinical studies in FLT3 mutation AML. Study of signal transductions and gene profile expression will contribute to further understanding of the drug mechanisms. *Acknowledgments.* COFIN 2005 (Myelodisplastic sindroms: pathogenetic models and promise of new therapies), COFIN 2003 (Molecular therapy of leukemias), by FIRB 2001, by the University of Bologna (60%), by the Italian Association for cancer research(A.I.R.C.), by the Italian National Research Council (C.N.R), by Fondazione Del Monte of Bologna e Ravenna (Italy) and A.I.L. grants.

PO-087

THE POLYMORPHISMS RAD51-G135C AND CYP3A4-A290G MAY INCREASE THE RISK OF ACUTE MYELOID LEUKAEMIA

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DNA is at constant risk from damage from both endogenous and exogenous sources and this damage causes chromosomal instability leading to oncogenesis, apoptosis and severe failure of cell functions. DNA is protected from damage by detoxification enzymes belonging to two different classes or damage triggering phases: phase I enzymes metabolize the exogenous agent to a reactive state, whereas phase II enzymes detoxify the reactive intermediate by catalysing conjugation. Even if greater activation and lesser detoxication of carcinogens result in DNA adducts, it is still possible for genomic integrity to be restored through DNA repair. Several polymorphisms in genes involving in both detoxification and repair pathways have been identified and many of them have been shown to influence the risk of developing solid tumours and haematological malignancies. The aim of our study is to investigate the frequency of polymorphisms involved in detoxification and double strand break (DSB) repair via homologous recombination (HR) pathways and to correlate them with AML or therapy-related AML (t-AML) risk. We studied 161 patients with AML (131 de novo and 30 therapy-related) and 161 control subjects, matched for age and sex. RFLP PCR were used to analyze genotypes of DNA repair genes for RAD51 (RAD51-G135C) and XRCC3 (XRCC3-Thr241Met) and detoxification genes for NQO1 (NQO1-Pro187Ser), GSTA1 (GSTA1-C69T) and GSTP1 (GSTP1-Ile105Val). The polymorphism in the promoter region of detoxification gene CYP3A4 (CYP3A4-A290G) was examined by mis-match PCR. Differences in genotypes distribution in the AML (*de novo* or therapy related) cases and the controls subjects were compared for statistical significance using the y2 test (Yates corrected). Odds ratios with 95% confidence intervals were also calculated for the genotypes. Comparing *de novo* AML patients to controls, a statistically higher prevalence of the g/c

Table (PO-087). Clinical characteristics, thrombophilia, etiology, treatment and outcome of children with cerebral sinovenous thrombosis.

Genotype	controls n (%)	AML			t-AML		
		n (%)	OR (95% CI)	p	n (%)	OR (95% CI)	p
RAD 51	161	131			30		
g/g	142 (88.2)	100 (76.3)	1.0 (Ref)		26 (86.7)		1.0 (Ref)
g/c	18 (11.2)	30 (22.9)	2.37 (1.20-4.71)	0.011	3 (10.0)	0.91 (0.16-3.46)**	1.000*
c/c	1 (0.6)	1 (0.8)	1.42 (0.02-112.24)**	1.000*	1 (3.3)	5.46 (0.07-431.61)**	0.293*
g/c & c/c	19 (11.8)	31 (23.7)	2.32 (1.19-4.54)	0.012	4 (13.3)	1.15 (0.26-3.86)*	0.764*
XRCC3-241	161	131			30		
Thr/Thr	48 (29.8)	43 (32.8)	1.0 (Ref)		12 (40.0)	1.0 (Ref)	
Thr/Met	93 (57.8)	56 (42.7)	0.67 (0.38-1.18)	0.180	11 (36.7)	0.47 (0.18-1.25)0.150	
Met/Met	20 (12.4)	32 (24.4)	1.79 (0.84-3.80)	0.141	7 (23.3)	1.40 (0.42-4.58)	0.735
Thr/Met & Met/Met	113 (70.2)	88 (67.1)	0.87 (0.51-1.47)	0.670	18 (60.0)	0.64 (0.27-1.54)	0.374
NQO1-187	155	128			29		
Pro/Pro	108 (69.7)	84 (65.6)	1.0 (Ref)		17 (58.6)		1.0 (Ref)
Pro/Ser	40 (25.8)	39 (30.5)	1.25 (0.72-2.19)	0.478	9 (31.0)	1.43 (0.54-3.75)	0.577
Ser/Ser	7 (4.5)	5 (3.9)	0.76 (0.18-3.04)**	0.898	3 (10.3)	2.72 (0.41-13.32)**	0.168*
Pro/Ser & Ser/Ser	47 (30.3)	44 (34.4)	1.20 (0.71-2.05)	0.549	12 (41.3)	1.62 (0.67-3.93)	0.340
GSTA1 Promoter	158	127			30		
c/c	62 (39.2)	57 (44.9)	1.0 (Ref)		14 (46.7)		1.0 (Ref)
c/t	68 (43.0)	62 (48.8)	0.99 (0.58-1.68)	0.925	15 (50.0)	0.98 (0.41-2.35)	0.881
t/t	28 (17.7)	8 (6.3)	0.31 (0.12-0.79)	0.011	1 (3.3)	0.16 (0.00-1.15)**	0.062*
c/t & t/t	96 (60.7)	70 (55.1)	0.91 (0.60-1.36)	0.692	16 (53.3)	0.74 (0.31-1.74)	0.577
CYP3A4 Promoter	159	130			29		
a/a	154 (96.9)	116 (89.2)	1.0 (Ref)		28 (96.6)		1.0 (Ref)
a/g	5 (3.1)	13 (10.0)	3.45 (1.11-12.67)**	0.030	1 (3.4)	1.10 (0.02-10.37)**	1.000*
g/g	0 (0.0)	1 (0.8)	Undefined**	0.432*		0 (0.0)	Undefined
Undefined							
a/g & g/g	5 (3.1)	14 (10.8)	3.72 (1.22-13.51)*	0.018	1 (3.4)	1.10 (0.02-10.37)*	1.000*
GSTP1-105	157	127			28		
Ile/Ile	92 (58.6)	64 (50.4)	1.0 (Ref)		16 (57.1)		1.0 (Ref)
Ile/Val	54 (34.4)	51 (40.2)	1.36 (0.80-2.30)	0.281	8 (28.6)	0.85 (0.31-2.29)	0.908
Val/Val	11 (7.0)	12 (9.4)	1.57 (0.60-4.11)	0.433	4 (14.3)	2.09 (0.43-8.20)**	0.265*
Ile/Val & Val/Val	65 (41.4)	63 (49.6)	1.39 (0.85-2.29)	0.207	12 (42.9)	1.06 (0.44-2.57)	0.949

**Fisher exact (2-Tailed), **Exact Confidence Limits.*

and g/c + c/c genotype of the DNA repair enzyme RAD-51 was found in AML patients (p=0.012). Similarly an higher prevalence of a/g and a/g + g/g genotype of the DNA detoxification enzyme CYP3A4 was found in *de novo* AML patients (p=0.018). The frequency of the t/t genotype in GSTA1 polymorphism was lower in AML patients, compared to controls (p=0.01), indicating a possible protective role. No differences were found when looking at XRCC3, NQO1 and GSTP1 polymorphisms. The comparison of genotype frequencies between t-AML patients and controls revealed no differences for analysed polymorphisms. The polymorphisms in the RAD51 DNA repair gene and in the CYP3A4 detoxification gene may increase the risk of developing acute myeloid leukaemia. When looking at association between detoxification and DNA-repair polymorphisms, the risk is significantly higher when the RAD51-G135C polymorphism is associated to the CYP3A4-A290G polymorphism.

PO-088

MOLECULAR QUANTIFICATION OF AML/ETO FUSION TRANSCRIPT FOR MINIMAL RESIDUAL DISEASE DETECTION AND PROGNOSTIC EVALUATION IN T(8;21) ACUTE MYELOID LEUKEMIA PATIENTS

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The t(8;21) translocation derives from the fusion of AML1 on chromosome 21 and ETO on chromosome 8. It is associated with FAB subtype M2 acute myeloid leukemia (AML). In order to predict relapse, qualitative PCR has a limited value since a positive PCR can be observed for a long follow-up period, even during continuous complete remission (CCR). From preliminary studies, quantitative RT-PCR seems to be a good candidate to predict clinical outcome of patients presenting AML1-ETO rearrangement. We tested the usefulness of quantitative RQ-PCR assay in detecting minimal residual disease and in predicting relapse in patients affected by t(8;21) AML. 123 PB and BM samples from 33 patients affected by AML presenting AML1-ETO rearrangement were analyzed in a retrospective manner using standard nested RT-PCR. 111 out of 123 BM samples were also analyzed by quantitative RQ-PCR technique following standard methods. Samples were taken at diagnosis, after induction and consolidation therapy. The median age of the patients was 34 years (range 6-60). 13 patients underwent conventional chemotherapy and 20 were treated with autologous or allogeneic bone marrow transplantation. WBC median value at diagnosis was 7905 (range 400-23300). 9 out of 21 patients who achieved and remained in CCR showed qualitative RT-PCR positivity after consolidation treatment, in two cases even after allogeneic and autologous transplantation showing a late clearance of the transcript. By contrast, 5 patients out of 12 achieved PCR negativity but subsequently relapsed. We compared the values obtained by RQ-PCR in the 21 patients who achieved complete remission to the 12 who relapsed during follow-up. At diagnosis quantitative analysis of AML-ETO fusion transcript showed a large variability (median value 71580, range 9632-801900). No difference was found between transcript amount at diagnosis and clinical outcome (p=0,6 by Mann-Whitney test). We did not observe any significant correlation between transcript amount and either WBC values (r=0,14) or blast percentage (r=0,23). Values obtained after induction treatment are not different in patients who reached CR as compared to those who relapsed (p=0,17). By contrast, after consolidation treatment, patients in CR showed a median transcript level of 14, compared to 393 AML-ETO copies in those who subsequently relapsed (p=0,014). We also tried to identify a threshold level of transcript after consolidation to identify the CCR patients. We can observe that only two patients out of 21 in CCR reached a post consolidation value > 15 copy numbers. On the other hand only 4 out of 20 patients who obtained a post consolidation value below 15 copies subsequently relapsed. Our data demonstrated that quantitative RT-PCR assessment of AML/ETO fusion transcript amount in leukemic patients is a useful tool for detecting minimal residual disease. Moreover, the most significant value to predict final outcome is the one obtained after consolidation treatment. We can also identify 15 AML-ETO copies after consolidation as threshold level in order to predict patients' outcome.

PO-089

NPM MUTATIONS: A POTENTIAL MARKER FOR MONITORING MINIMAL RESIDUAL DISEASE IN THE ACUTE MYELOID LEUKEMIA

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Nucleophosmin (NPM) is a multifunctional phosphoprotein that acts as nucleocytoplasmic shuttling protein, with tumour suppressor and oncogenic functions. Recently, NPM mutations have been found in a subset of adults with de novo acute myeloid leukemia (AML). These mutations occur in the last coding exon (exon 12), causing a frameshift and the formation of novel C-termini. The abnormal mutated NPM protein shows aberrant cytoplasmic localization and is frequently associated with FLT3 mutation. These observations provide basis for studies of the pathogenesis in AML. We did sequential analysis on patient samples during the clinical course to investigate the stability and pathogenetic role of NPM mutation in AML and the association with FLT3 mutations. The NPM mutations were determined by D-HPLC analysis and samples exhibiting an abnormal D-HPLC profile were confirmed by direct sequencing. In this study we analyzed NPM mutations in 11 patients of de novo AML. We found that NPM mutations were present in 9 patients at diagnosis; 6 patients harboring NPM mutation presented mutant FLT3. The cases with wild type NPM not showed mutant FLT3. Analysis of NPM mutations showed the mutation disappeared at complete remission, but the mutation reappeared at relapse, except for one who lost the mutation at relapse. Furthermore, we observed that FLT3 mutations showed the same course of NPM mutations in the disease evolution of 8 patients. (see Table). These results suggest that NPM mutations may have utility as a potential marker for monitoring minimal residual disease. Studies on the biological effects of NPM mutations will contribute to disclose the role of NPM mutations in the pathogenesis of AML and their interactions with other genetic alterations such as FLT3.

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Table.

ID	*STATUS*	*NPM mutation*	*FLT3 mutation*
1	Diagnosis	+	+
1	relapse	+	+
2	Diagnosis	+	-
	I relapse	+	+
3	Diagnosis	+	+
	I relapse	+	+
	II RC	-	-
	II relapse	+	+
4	Diagnosis	+	+
	I relapse	-	-
5	Diagnosis	+	+
	I relapse	+	+
6	Diagnosis	+	+
	I RC	-	-
	I relapse	+	+
7	Diagnosis	-	-
	I RC	-	-
8	Diagnosis	+	+
	I RC	-	-
	I Relapse	+	+
9	Diagnosis	-	-
	I RC	-	-
	I relapse	-	-
10	Diagnosis	+	-
	I relapse	+	-
11	Diagnosis	+	-
	I relapse	+	+

PO-090

FAS-L-MEDIATED APOPTOSIS OF CD8+ LYMPHOCYTES IN ACUTE MYELOID LEUKEMIAS: RELATIONSHIP WITH INCREASED SERUM SOLUBLE HLA-I

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There is increasing evidence for the presence of soluble HLA-I (sHLA-I) molecules in the serum and urine of healthy individuals. More recently, however, it has been shown that the serum level of these soluble molecules is significantly increased in patients with an activation of their immune system, such as during allograft rejection, acute graft versus host disease after bone marrow transplantation, autoimmune diseases or viral infections. Moreover, sHLA-I molecules can be released by tumor cells, and high sHLA-I serum levels have been found in solid cancers, melanomas and lymphomas. Thus, sHLA-I molecule are not specific markers for organ rejection, but rather are affected by inflammatory processes and viral or neoplastic transformation. In this study, we show that high serum levels of soluble HLA class I molecules (sHLA-I, range: 0.7-1.7mg/mL) and soluble Fas ligand (FasL, range: 0.4-1.9ng/mL) are detected in patients with acute myeloid leukemia (AML) at diagnosis, compared to healthy donors (sHLA-I range: 0.1-0.6 mg/mL; sFasL range: 0.1-0.4 ng/mL). Both sHLA-I and sFasL serum concentrations increased during chemotherapy. The functional role of sHLA-I molecules either in physiological or in pathological conditions is not clear: it has been described that HLA-I molecules can trigger cytotoxic T lymphocytes to release cytolitic enzymes and pro-inflammatory cytokines. However, we and others reported that sHLA-I molecules bind to CD8 receptors expressed on cytotoxic effector lymphocytes leading to activation-induced apoptosis or cell death mediated by synthesis and secretion of FasL and the consequent interaction with Fas expressed by T and NK cells. AML patients' sera were able to induce transcription and secretion of FasL in CD8+ T cells, followed by apoptosis *in vitro*; this apoptosis was inhibited by either anti-HLA-I or anti-FasL specific monoclonal antibodies. These findings closely relate to the *in vivo* up-regulation of FasL transcription observed in peripheral blood lymphocytes from AML patients; in the same cells, mRNA for the antiapoptotic protein Bcl-2 was down-regulated. Interestingly, in AML patients caspase-8 and caspase-3, both downstream mediators of death receptors-induced apoptosis, were activated *in vivo* in CD8+ cells, that were already committed to apoptosis, at variance with CD4+ cells and cells of healthy donors. These data strongly suggest that in AML, increased levels of sHLA-I molecules may be responsible for the elimination of potentially anti-tumor effector cells through a FasL/Fas interaction.

PO-091

BAALC (BRAIN AND ACUTE LEUKEMIA CYTOPLASMIC) EXPRESSION LEVELS IN ACUTE MYELOID LEUKEMIA (AML)

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Clonal chromosome abnormalities are discovered in about 40-60% of AMLs and play a major role in the diagnostic work-up and prognostic stratification of patients. In the remaining cases conventional cytogenetics shows a completely normal karyotype and does not provide any information. These chromosomally normal patients are supposed to have an intermediate prognosis even though only 40% of them become long-term survivors. For this reason in these patients new molecular markers effecting clinical outcome have been intensively searched in order to better define their prognosis. Recent data suggest that the level of expression of the BAALC gene may be one of the most relevant. We analysed BAALC expression in the pre-treatment bone marrow samples from 28 adult AMLs (8 M0-M1, 3 M2, 16 M4-M5 and 1 M6). They were 9 females and 19 males, their median age was 56 years (range 21-75). A normal karyotype was discovered in 13 patients, a del(7)(q31q35) in 2, a del(5)(q23q33) [one with +8] in 2, a complex karyotype (more than 3 abnormalities) in 2, an inv(16)(p13q22) [two with +8] in 6, and miscellaneous defects in 3. Our aims were to detect the incidence of high BAALC expression in AML patients and to correlate high BAALC expression with clinical/biological parameters and outcome. Statistical analysis were carried out by applying the method created by Pfaffl HW & Dempfle L (Nucleic Acids Res 2002;30:536). BAALC relative quantification was obtained through a real-time PCR which used SybrGreen I as a double-stranded DNA-binding fluorescent dye. The forward and reverse primers used were those already published by Baldus et al (JCO 2006;24:790). Serial dilutions of total RNA from an AML patient exhibiting high BAALC expression levels were carried out in order to design the standard curve for real-time quantification. Quantification was achieved by applying the DDCt method. BAALC expression was normalized to the ABL1 gene and calibrated on a normal control sample. A reference interval for BAALC expression quantification was fixed at 0,609 (mean expression [0,207]±three times the standard deviation [0,134]) after having analysed 12 normal controls. BAALC expression was low in 13 patients (median±3SD =0,158±0,561) and high in 15 (median±3SD = 5,427±11,691) with a statistically significant difference (p=0,001). The two patients group showed no difference in age, sex, white blood cell count and percentage of bone marrow blasts. A high BAALC expression was predominantly observed in M4-M5 cytotypes. Considering the chromosome pattern, a low BAALC expression was discovered in 6/13 chromosomally normal patients, while a high expression in all the 4 patients with a single inv(16) and in all the 3 with +8. Ten patients with low BAALC expression were submitted to induction chemotherapy: 7 obtained a complete remission (CR) and 3 did not respond. Five of the 7 CRs relapsed and did not achieve a second CR. Twelve patients with high BAALC expression were submitted to induction chemotherapy: 9 achieved CR and 3 did not respond. Five of the 9 CRs relapsed but succeeded in achieving a second CR. In our study a high BAALC expression was observed in 53% of patients; trisomy 8 and inv(16)(p13q22) were the chromosome defects strongly associated with a high BAALC expression; CR duration and overall survival were longer in patients with high BAALC expression than in those with low BAALC expression perhaps because of the higher occurrence of +8 and inv(16) in the first patients group.

PO-092

SU11657, A NOVEL FLT3 INHIBITOR, SHOWS BIOLOGIC ACTIVITY IN ACUTE MYELOID LEUKEMIA (AML) CELLS *IN VITRO*

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fms-related tyrosine kinase3 (FLT3) is one of the most commonly mutated gene in human acute myeloid leukemia (AML) and has implicated in its pathogenesis. Constitutive activation of the FLT3 receptor tyrosine kinase, has been linked either by internal tandem duplication (ITD) of the juxtamembrane region or by point mutation in the second tyrosine kinase domain (TKD). The purpose of the study was to evaluate, *in vitro*, the effect and the biological activity of SU11657 (Pfizer), a new compound FLT3 kinase inhibitor. SU11657 was investigated on human cell lines from AML patients (MV4-11 and HL-60) and blast from patients AML using a wide range of concentrations (1nanomolar-10 micromolar). FLT3 expression levels were evaluated by flow cytometry. Furthermore, to evaluate the effect of SU11657 we analyzed the cytotoxicity, induction of apoptosis and inhibition of cell proliferation by flow cytometry. The antiproliferative and cytostatic effects of SU11657 were confirmed by analysis of signal transduction. HL-60 cell line served as a control as it expresses a wild type receptor. MV4-11 is a cell line that expresses a naturally internal tandem duplication (-ITD) in homozygous form. In HL-60 does not show relevant effect after treatment with SU11657. Instead, in MV4-11 we observed a decrease dose-dependent in cell viability after treatment with SU11657. The effects of this compound on cell cycle progression show an accumulation of G1/S phase and an induction of apoptosis at 1-10nanomolar concentration after 24h of treatment. First we observed a dephosphorylation of FLT3 on Tyr(591) in whole cell extracts from MV4-11 cells after treatment with SU11657 100nanomolar. We also demonstrated a hypophosphorylation of AKT on Ser(473) and a consequently dephosphorylation of BAD on Ser(136) at nanomolar concentration. We observed a dephosphorylation of STAT-5 to 100nanomolar of Su11657 at 24h. We evaluated the effects of this

new compound in AML primary progenitors that showed FLT3-ITD, FLT3-TKD and FLT3-wt. In the patients with mutation ITD and TKD was evident a modification of cell cycle progression with a decrease in G2/M phase and an increase of subdiploid peak. The effect of SU11657 in patients FLT3-wt was not relevant. Due to its FLT3 inhibitory activity, SU11657 represent promising compound for clinical studies in FLT3 mutation AML. Study of signal transductions and gene profile expression will contribute to further understanding of the drug mechanisms.

COFIN 2005 (Myelodisplastic sindroms: pathogenetic models and promise of new therapies), COFIN 2003 (Molecular therapy of leukemias), by FIRB 2001, by the University of Bologna (60%), by the Italian Association for cancer research(A.I.R.C.), by the Italian National Research Council (C.N.R), by Fondazione Del Monte of Bologna e Ravenna (Italy) and A.I.L. grants.

PO-093

FLT-3 ACTIVITY AND ITS RESPONSE TO DRUGS CAN BE DETERMINED IN AML BLAST CELLS BY FLT-3 PHOSPHORYLATION STATUS USING FLOW CYTOMETRY

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One of the most common molecular defects identified in acute myeloid leukemia (AML) patients is an activating mutation of FLT3 tyrosine kinase. The identification of activated FLT3 as a contributor to the cause and progression of much leukemia has led to its consideration as a potential target for therapy. Since small molecule FLT3 kinase inhibitors are actually in clinical trials; a robust and standardized method for screening of FLT3 receptor activation is necessary. We evaluated the expression level of FLT3 receptor (CD135) by Facs analysis. We developed a flow cytometry method to measure FLT3 phosphorylation (P-FLT3) in samples with $<10^5$ cells. The amount of P-FLT3 in the samples was determined as the mean fluorescence intensity (MFI). The P-FLT3 status of the treated samples was expressed as a percentage of the untreated control (100%). The method was first validated in FLT3 wild-type (HL-60) and mutant (MV4-11/ITD+) as well as FLT3 negative (K562) cell lines. The method also provides to be reproducible with samples AML from patients. Analysis was performed after exposure to drugs, *in vitro* and *in vivo*. In response to increasing drugs concentration (CEP-701 and SU11657) there was a linear reduction in P-FLT3. The results validate a rapid method to detect P-FLT3 protein at the single cell level by flow cytometry, and enable an accurate assessment of FLT3 kinase activity in blast cells in response to novel tyrosine kinase inhibitors. *Acknowledgments.* COFIN 2003 (Molecular therapy of leukemias), by FIRB 2001, by the University of Bologna (60%), by the Italian Association for cancer research(A.I.R.C.), by the Italian National Research Council (C.N.R), by Fondazione Del Monte of Bologna e Ravenna (Italy) and A.I.L. grants.

Acute Myeloid Leukemia II

PO-094

CORRELATION BETWEEN THE RESPONSE TO INDUCTION TREATMENT, INCLUDING OR NOT FLUDARABINE AND THE MDR PHENOTYPE IN NEWLY DIAGNOSED ACUTE MYELOID LEUKEMIA PATIENTS. RESULTS OF A CASE-CONTROL STUDY

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The multidrug resistance (MDR) mediated by the overexpression of the 170-kd trans-membrane P-glycoprotein (Pgp), is known as one of the most relevant causes of treatment failure in acute myeloid leukaemia (AML). Fludarabine is a myelotoxic agent, synergistic with AraC and not exported by Pgp-MDR protein. One hundred and six newly diagnosed AML patients, younger than 60 years, who were enrolled and treated with fludarabine + Ara C + idarubicine (FLAI) in two consecutive multicenter studies between January 1997 and January 2005 (cases), were matched for the age, cytogenetics, MDR phenotype and white blood cells (WBC), with 106 newly diagnosed AML patients consecutively treated between July 1991 and January 2005 with a non-fludarabine containing regimen, mainly idarubicin/daunorubicin + AraC +/- etoposide (controls). Out of 212 patients, 143 (63%) were Pgp-MDR- and 78 (37%) were Pgp-MDR+. For what concern the response, a significant overall advantage in favour of the cases treated with FLAI was observed (p= 0.0243), since 74/106 cases (70%) vs 62/106 controls (58%) obtained a CR and 2/106 cases (2%) vs 11/106 controls (10%) died during induction. The non-response (NR) rate was similar in the two groups. Focusing the analysis according to the Pgp-MDR phenotype, a trend towards a higher CR rate in the cases with respect to the controls was observed, both in MDR+ and MDR- patients. The lowest CR rate was obtained in the MDR+ patients treated with a non-fludarabine containing regimen (controls) (17/39, 44%). By univariate analysis, the MDR+ phenotype (p=0.0073), the karyotype group (p=0.0169) and the age (p=0.0247) were negatively correlated with CR. The non-fludarabine treatment arm appeared to be a negative prognostic factor with a border-line significance (p=0.0857). By multivariate analysis, the negative prognostic factor independently correlated with CR was the MDR+ phenotype [Odds ratio (OR) MDR+ vs MDR- 2.23; 95% CI 1.24-3.99; p=0.0074]. The non-fludarabine treatment arm appeared to be a negative prognostic factor with a border-line significance [OR 0.599; 95% CI 0.336-1.066; p=0.0815]. The risk of no CR in the MDR+ controls was higher than in the MDR+ cases [0.53 (95% CI 0.406-0.664) vs 0.41 (95% CI 0.289-0.543)], with a relative risk of no CR for patients with MDR+ of 1.31. The results of this case-control study confirm the prognostic impact of the Pgp-MDR phenotype in AML, both in univariate and multivariate analysis. The induction treatment with FLAI showed to be more effective and moreover less toxic than non –fludarabine containing regimens. Although this superiority was not statistically significant, a trend was observed in MDR- patients and moreover in MDR+ patients. Further prospective randomized trials based on a larger number of patients are warranted to confirm these results.

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PO-095

COMPARISON OF LEUKEMIC CONTAMINATION IN PERIPHERAL BLOOD STEM CELL LEUKAPHERESES AND BONE MARROW AFTER CONSOLIDATION THERAPY IN ACUTE MYELOID LEUKEMIA

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Background. Leukemic relapse is the most frequent cause of treatment failure in acute myeloid leukemia (AML) patients after autologous stem cell transplantation (ASCT). The use of the peripheral blood stem cells

(PBSC) instead of the bone marrow (BM) might increase the probability of leukemic contamination and relapse due to the large quantity of reinfused cells. Aim of the study was to evaluate the presence and the prognostic significance of residual leukemic cells after consolidation therapy in AML patients presenting with cytogenetic and/or molecular abnormalities at diagnosis. MethodsBetween January 1994 and May 2006 we investigated by means of cytogenetic and/or a nested RT-PCR analysis the leukemic cell contamination in PBSC leukaphereses and in BM in 46 AML patients in morphological complete remission after consolidation therapy. Median age was 40.8 years (range 10-65). Karyotypic analysis revealed t(15;17) in eleven patients, t(8;21) in eleven patients, inv16 in eleven patients, t(9;22) in three patients; other abnormalities (t(6;11), t(6;17), 6p-, -18 + m, -5+10+18+2m, t(4;16), del5) in 7 patients. Molecular rearrangements were the following: PML/RARα in 11 patients, AML1/ETO in 13 patients, inv16 in twelve patients, BCR/ABL (P190) in three patients, MLL/AF6 in one patient. Thirty-six cases (group A, 78.3%) expressing t(15;17), inv16 or t(8;21) were classified as favourable cytogenetic group whereas 10 patients (group B, 21.7%) had high-risk cytogenetic and molecular characteristics. *Results.* PBSC were harvested in remission after the first consolidation course and a median number of CD34+ cells of 11.1×10^6/kg (range 1.3-57.7) was collected. The persistence of leukemic contamination was detected in PBSC as well as in BM (PBSC+/BM+) in 8 cases (17.4%), only in the BM (PBSC-/BM+) in 11 cases (23.9%), while no residual contamination (PBSC-/BM-) was detected in 27 cases (58.7%). Thirty patients performed ASCT (3 BM, 27 PBSC), 3 patients were given allogeneic transplantation. PBSC were not reinfused in 13 patients because 5 patients received post-consolidation high dose Ara-C therapy, 3 AML-M3 patients received maintenance with Gentuzumab Ozogamicin, 3 patients were in early relapse and 2 patients are too early. The outcome in PBSC+/BM+ patients versus PBSC-/BM+ and PBSC-/BM- patients was significantly different for overall survival (OS) (24.2±12.7 vs 65.9±16.3 vs 86.9±13.1 months respectively, log rank test 7.56, p 0.006) but not significantly different for disease-free survival (DFS) (32.1±15.7 vs 49.9±16.4 vs 60.8±13.0 months respectively, log rank test 2,97, *p* 0.08) Multivariate analysis by Cox proportional hazard method including age, sex, cytogenetic group at diagnosis, number of CD34+ harvested cells, contamination status, type of post-consolidation therapy, revealed that only cytogenetics was an indipendent and powerful indicator of clinical outcome (RR 18,18; 95% CI3.81-86.70). *Conclusions.* We found that PBSC are less frequently contaminated than BM. Our data show a close relationship between persistence of disease-related clone in harvested stem cells, adverse karyotype and poor prognosis. The presence of residual disease has a critical role in the high-risk group, while ASCT seems able to eradicate residual leukemia in the favourable cytogenetic group.

PO-096

POTENTIAL ANTITUMOR ACTIVITY OF BORTEZOMIB IN HUMAN ACUTE MYELOID LEUKEMIA

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The proteasome inhibitor Bortezomib has a documented antitumor activity in multiple myeloma and other lymphoid malignancies. Acute myeloid leukemia (AML) is a malignant disease characterized by abnormal proliferation of clonal myeloid precursor cells. Although different strategies have been performed to obtain complete remission, disease progression actually occurs in about 30-40% of patients. Here we examined the sensitivity to Bortezomib of bone marrow cells from newly diagnosed or relapsed/refractory AML patients (30 patients: 20 newly diagnosed, 4 relapsed, 5 refractory patients). Immunohistochemistry or immunofluorescence using a monoclonal mouse anti-human p65 (Rel A) showed that localization of NFκB was in the nucleus of AML blasts and it remained there after bortezomib exposure. In each sample Bortezomib was able to induce cell death of AML blasts. The cytotoxic effect was dose and time-dependent (concentration of Bortezomib ranging from 1 nanomolar to10 micromolar for 24 and 48 hours) and was associated with downregulation of Bcl-xL and upregulation of p21. Remarkably, antitumor activity was also obtained *in vivo*, in a relapsed/refractory AML patient that achieved a late partial response after 4 cycles of Bortezomib treatment. Bortezomib was administered the first cycle in combination with doxorubicin and dexamethasone (PAD combination therapy), then alone. In this patient, the bone marrow blast count was reduced from a baseline of 68% to 2,9% and remission was maintained for about 4 months. These findings suggest novel potential strategies of treatment for AML patients refractory to conventional chemotherapy.

PO-097

ACUTE MYELOID LEUKEMIA IN THE ELDERLY, INTENSIVE OR MAINTENANCE THERAPY? OUR EXPERIENCE IN PATIENTS OVER 65 YEARS

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The treatment of acute myeloid leukaemia in elderly with age > 65 years is still debated. In literature numerous studies have valued the feasibility of intensive chemotherapy in these patients. The aim of the study is to value the difference in EFS and OS among 2 groups of AML elderly patients treated with intensive chemotherapy (IC) or maintenance (M). From June 2001 to May 2006 we have treated in our Division 54 AML patients, 30 male and 24 female with median age of 73 years (66-90 years). 27 patients (16 M and 11 F with median age of 71 years) have received intensive chemotherapy (I.C. Flag and MICE) and 27 (14 M and 13 F with median age of 78.5 years) have received maintenance (low dose cytarabine and/or support). In IC group 12 patients (45%) have obtained to complete remission (CR) with to EFS and OS media of 4, 47 and 7, 15 months respectively, the rate of TRM has been of 25%. In the M group the CR has been documented in 8 patients (30%) with to EFS and OS media of 4,22 and 4,94 months respectively (Figure 1-2). This results have shown a best rate of CR in the IC group but the OS and EFS difference is not statistically significant in the two groups (*p*: 0.7). In conclusion the Intensive chemotherapy has not improved the survival in AML elderly patients. New therapeutics strategy is necessary for to improve the EFS and OS in these patients. Interesting is the use of specific monoclonal antibodies (anti CD33) in this poor disease especially in maintenance after a CR obtainable with an intensive or low dose chemotherapy.

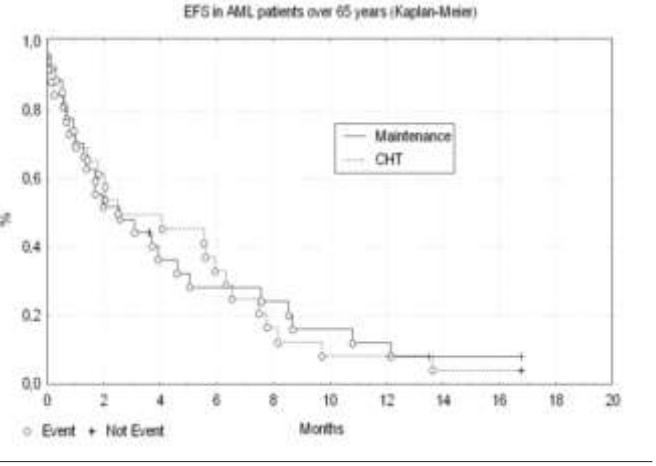

Figure 1.

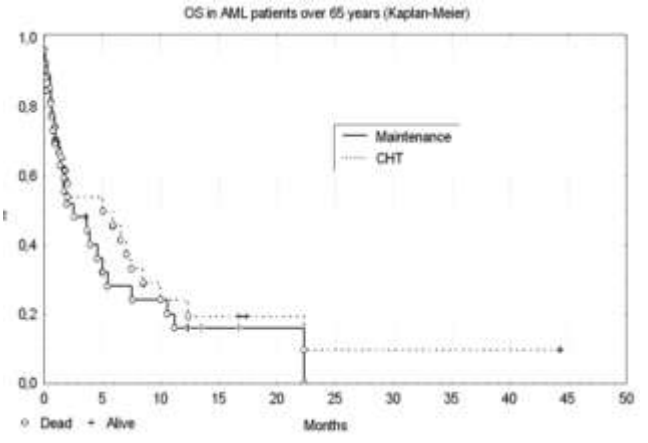

Figure 2.

PO-098

IMMUNOPHENOTYPIC DIFFERENCES BETWEEN DIAGNOSIS AND RELAPSE IN ADULT AML

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Immunophenotypic analysis of acute myeloid leukemia (AML) is useful to better characterize the lineage of the leukemic clone, to detect the expression of some antigens associated with prognosis, and to identify any *aberrant leukemic pattern* useful for the detection of minimal residual disease (MRD). However, changes in the antigen expression pattern between diagnosis and relapse have been reported with a frequency of 38-75% in patients with acute lymphoblastic leukemia and 62-91% in patients with AML. In this study we compared the immunophenotypic features of leukemic blast cells from patients with AML at diagnosis and first relapse by flow cytometry in order to detect losses or gains of antigens and their impact on the evaluation of MRD. The study was performed in 48 adult patients (23 males, 25 females) with a median age of 47 years (range 15-76); the distribution according to the FAB classification was as follows: M0 (2 cases), M1 (5), M2 (18), M3 (5), M4 (9), M4eo (2), M5a (5), M5b (2). Flow cytometric analysis was performed with triple staining on a FACScan flow cytometer using Cell Quest software; blasts were identified according to their side scatter profile and CD45 expression (CD45 dim cells) in order to include only leukemic cells in the gate of analysis, even in cases with a low percentage of blasts. Immunophenotypic changes occurred in 36 cases (75%) and involved from 1 to 6 antigens. Changes in progenitor-associated antigens (CD34, CD117) included more losses than gains (23% vs. 12%) whereas changes in lymphoid-associated antigens (CD7, CD10, CD19, CD56) included more gains than losses of antigen expression (29% vs. 10%); changes in myeloid antigens (CD11b, CD13, CD14, CD15, CD33, MPO) were almost balanced for loss and gain (25% vs. 35%). Four of 5 cases with t(8;21) underwent changes in antigen expression related to this translocation; particularly, we observed loss of CD19 in 1 case, gain of CD19 in 2 cases and gain of CD19 and CD56 in 1 case; notably, no change was observed in all M3 cases. Morphologically and by cytochemistry (PAS, PEROX, ANAE), differences were identified leading to changes in the FAB subtype in 8 cases. Particularly, 1 M1 case relapsed as M2, 1 M2 as M1, 1 M2 as M6, 3 M4 as M2, 1 M5a as M2 and 1 M5b as M5a; notably, 2 of 3 M4 cases relapsing as M2 were AML1/ETO positive at diagnosis and no additional chromosomal abnormality was observed at relapse. If we considered the intervals between complete remission and relapse we did not observe differences in terms of loss or gain in the three groups. A high frequency of immunophenotypic changes in antigen expression between diagnosis and relapse in AML patients seems to indicate either intrinsic instability of the leukemic clone, or induced by multiple drugs, and suggests the risk of false negatives in the detection of minimal residual disease, particularly if panels with limited numbers of antigens are used.

PO-099

PROTEOMIC MODIFICATIONS IN AML1-ETO POSITIVE AML BLASTS TREATED WITH AZACITIDINE.

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AMLs frequently present molecular alterations affecting core binding factor genes. The AML cell line Kasumi-1 expresses AML1-ETO fusion protein as result of the translocation t(8;21), leading to permanent recruitment of DNA methyltransferases, histone acetyl transferases and corepressor complex to DNA, inducing block of maturation. Ipomethylating agents represent a class of drugs with activity in the treatment of AMLs and other malignancies, but their action on AML induction of maturation has to be analysed. We studied the proteome modifications in Kasumi-1 cells treated with azacitidine as ipomethylating agent. Cells were cultured in presence of 1milliM azacitidine for 6 to 24 hours. Cytomorphological observation and cell cicle analysis by propidium iodide assay were conduced prior to proteomic analysis to monitor the effects of the treatment. Total cell protein extracts of treated and untreated cells were separated by two dimensional electrophoresis (2DE) on non-linear pH gradient 3-11. 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 treated and untreated cells. MALDI-TOF MS analysis (PMF and/or MS/MS experiments) of the most representative protein spots indicated that the correspondent identified proteins are involved in regulation, cellular processes and metabolic pathways. Moreover, common proteins with different spot distribution were identified, leading to the hypothesis of the presence of different isoforms of the same protein. Further analysis of different classes of HDACis may clarify whether acetylation of target proteins is critical and whether proteomic modifications can be identified as characteristic for each drug.

PO-100

SERUM TRYPTASE LEVELS IN ACUTE LEUKEMIA AT DIAGNOSIS: A MULTICENTRE RETROSPECTIVE STUDY

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α and β-tryptase genes cluster on the short arm of human chromosome 16 and encode lineage-associated serine proteases that are abundantly expressed in mast-cells and, in trace amounts, in basophils. Under physiologic conditions no other myeloid cells express tryptases. However, in several myeloid leukemia cell lines and in AML blasts, the level of tryptase is elevated. In an attempt at correlating the levels of tryptase at diagnosis with cytogenetic features, we analyzed serum samples from 150 AML and 57 ALL adult patients. The total serum concentration was determined by UniCAP 100 and UniCAP Tryptase Fluorenzyme Immunoassay Kit (Pharmacia-Upjohn, Uppsala,Sweden). The median value of tryptase level in the control group (50 healthy people; mean age 35-y, range 20-50; M/F= 26/24) amounted to less than 5 nanograms/milliliter, ranging from 1 to 15 nanograms/milliliter. We detected elevated tryptase levels (more than 15 nanograms/milliliter) in 66 out of 150 AML-patients (44%) and in 1 out of 57 ALL-patients (1.75%; median value 1.2 nanograms/milliliter) (p=<0.0005, Fisher's exact test). In AMLs, by means of Bernouilli simulation, we demonstrated that elevated tryptase values are significantly bound to patients with t(8;21) (n = 27, p = 0.002)but not to inv(16) (n=17, p=0.071). Furthermore, we found a significant association between tryptase < 15 nanograms/milliliter and patients with normal karyotype (n=58; p=0.000015), t(15;17) (n=12, p=0.019) and additional chromosome 8 (n= 6, p=0.015). In conclusion, data suggest that elevated serum tryptase levels at diagnosis should be considered as a useful novel marker for t(8;21) AML.

PO-101

SAFETY AND EFFICACY OF THE FOUR DRUG INDUCTION REGIMEN MY-FLAIG-3 (MYLOTARG, FLUDARABINE, CYTARABINE AND IDARUBICIN) FOR ELDERLY ACUTE MYELOID LEUKEMIA PATIENTS. RESULTS OF A PHASE II PILOT STUDY

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Despite significant progress in acute myeloid leukemia (AML) treatment in the last decades, the outcome of elderly patients is still dismal. The addition of either fludarabine or mylotarg to the classical combination of antracyline and cytarabine (3/7 regimen and similar) has been proposed in order to improve the clinical outcome, without success until now. *Aim of the study.* We evaluated the safety profile and the efficacy of a 4 drug induction regimen (My-FLAIG-3) in elderly AML patients. *Methods.* Twenty-seven AML patients (15 males) were enrolled. Their median age at diagnosis was 64 years (51-75). Patients received the combination of fludarabine (25 mg/sqm/day for 3 days), cytarabine (1 g/sqm/day for 3 days), idarubicine (5 mg/sqm/day for 3 days), and mylotarg (3 mg/sqm day +4) as induction therapy. In addition, 24 patients received G-CSF (5 microgrammi/day starting on day +9, until hematological recovery) and 3 pegylated G-CSF (6 mg on day 9). In case of CR, an identical consolidation course was administered. Fourteen out of 27 patients had a secondary AML, 9 had an unfavorable cytogenetics, 20 were older than 60 years, and 5 had peripheral blast count higher than 30×10^9/L. *Results.* Twelve out of 27 patients (44.4%) achieved complete remission (CR) after a single induction course; 4/27 patients died during treatment (14.8%). Eleven patients also received the consolidation course, while 1 did not yet. Ten out of 12 responders actually relapsed. Nineteen patients died of disease, and 1 for myocardial stroke while in CR; 7 are currently alive (2 in CR). The median relapse free survival (RFS) and overall survival are 7.4 and 9.4 months respectively. All the patients developed grade IV hematological toxicity. Eighteen patients presented with fever during treatment period (fever of undetermined origin (FUO), N=4; Gram+ bacteremia, N=9; possible lung fungal infection, N=4; microbiologically documented aspergillosis of maxillary sinus, N=1). Gastro-intestinal toxicity (anorexia, nausea, vomiting, mucositis) was generally mild and only one patient developed a grade III WHO hemorrhagic syndrome (melena). The median time to ANC recovery (> 1.0×10^9/L) was 26 days (range, 17-57). The median time to PLT recovery (> 100×10^9/L) was 31 days (range, 19-46). *Conclusions.* The present study suggests that My-FLAIG-3 is a feasible induction regimen in elderly AML patients, with an acceptable toxicity profile. However, the clinical outcome in terms of CR rate and RFS seems to be not superior when compared to historical controls treated with 3/7. New molecular targeted therapies are probably necessary in order to improve these results.

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PO-102

INDETERMINATE CELL HISTIOCYTOSIS EVOLVING INTO ACUTE MYELOGENOUS LEUKAEMIA

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Introduction. Cutaneous indeterminate cell histiocytosis (ICH) is a mainly benign disorder of the *indeterminate cells* (ICs), CD1a (+) and $S100^+$ dendritic skin elements, similar to Langerhan cells (LC) not containing Birbeck granules (by electron microscopy) and immunohistochemically langerin/$CD207^-$. *Materials and methods.* We describe the case of a 39-year-old man who developed cutaneous ICH characterized by an infiltrate of histiocytic cells with abundant cytoplasm and irregularly-shaped clefted nuclei, that were immunohistochemically CD1a, CD1b, CD1c, CD11b (partially), CD11c, CD14, CD15, CD68, CD123 (partially), S100, MAC387 and HLA-DR positive, and CD3, CD4, CD16, CD33, CD34, CD36, CD56, CD207/langerin, Factor XIIIa and TCR β F1 negative; T-cell receptor-γ and immunoglobulin JH gene rearrangements were polyclonal. Ultrastructural analysis confirmed the dendritic morphology of the infiltrating cells (Birbeck granule-negative). Clinical staging showed no evidence of extracutaneous disease. Numerous relapses requiring different cytotoxic courses occurred over a 5-year follow-up period until the patient presented fever, mild productive cough, and dyspnea. *Results.* Computed tomography showed complete interstitial and nodular infiltration, of the lung parenchyma. The lung biopsy specimen was compatible with acute myelogenous leukemia (AML) invasion. The alveolar spaces were completely filled by a blastic population of medium-large cells, that sometimes had irregular and convoluted nuclei and a small amount of amphophilic cytoplasm. The neoplastic cells were $CD45^+$, $CD45Ro^+$, $Vimentin^+$, $CD68/PG\text{-}M1^\pm$, $CD34^\pm$, $CD1a^-$, $CD3^-$, $MPO^\pm$, and $S100^-$, thus suggesting a myelomonocytic lineage [Ki-67: 30-40%]; the T cell receptor-γ and immunoglobulin JH gene rearrangements were polyclonal. A bone marrow aspirate was consistent with the diagnosis of AML, French-American-British (FAB) type M4. Immunophenotypic analysis confirmed the blast cells' myelomonocytic nature [$CD45\ dim^+$, $CD33^+$, $CD15^+$, $CD14^+$, $CD117^-$, $CD34^-$, MPO^+, $HLA\text{-}DR^+$] [cytogenetic analysis: t (1;8) (q21; q13); del. chr. 6 (q14; q16); del. chr. 9 (q13; q33)]. A cell line was also established from the bone marrow mononuclear cells. The patient died during induction chemotherapy. *Conclusions.* In comparison with the immunophenotype of the initial cutaneous neoplastic infiltrate, the monocyte/macrophage phenotype profile of the blastic cells and cell line may suggest a case of unfavourable AML evolving from a rare subtype of ICH. It may be wise to limit chemotherapy to advanced ICH and closely monitor a patient's course in order to prevent clinical complications, particularly the onset of new hematological disorders.

PO-103

DIAGNOSIS OF INVASIVE ASPERGILLOSIS BY TRACKING ASPERGILLUS-SPECIFIC T-CELL IN NEUTROPENIC PATIENT WITH PULMONARY INFILTRATES

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Invasive aspergillosis (IA) is a leading cause of infection-related mortality in hematologic patients, because of the difficulties in a timely and undoubted diagnosis. The current diagnostic tools are limited by multiple factors. Recent studies have been shown that T-cells contribute to the host defence against Aspergillus species, that a high ratio of antigen-specific interferon-γ producing T-cells (IFN-γ-T h1) to antigen-specific inteleukin-10 producing T-cells (IL-10-Th2) is predictive of surviving IA, and that adoptive Aspergillus-specific IFN-γ-Th1 infusion may cure IA in allogeneic bone marrow transplant (alloBMT) patients. To evaluate whether enumeration of Aspergillus-specific IFN-γ-T h1 and IL-10-Th2 through an ex vivo enzyme-linked immunospot (ELISPOT) assay could improve the diagnosis and the clinical management of IA, we report the first clinical application of ELISPOT in a series of 7 consecutive neutropenic patients with pneumonia. Six patients had an hystologically or microbiologically proven pneumonia of non fungal etiology, the seventh patient had a proven IA. The latter was a 59 year-old AML patient, who needed a video-assisted-thoracoscopic resection of an ovalar nodule surrounded by a halo of ground glass opacity in the left lung, developed during neutropenia of induction chemotherapy. Hystologic, immunohystochemical and cultural examination disclosed IA. Serial cultural, serologic and molecular examination of blood, urine, feces and bronchoalveolar lavage fluid has been resulted repeatedly negative for bacterial, fungal or viral pathogens. In this patient ELISPOT resulted positive for IL-10-Th2 at each determination, and showed an increasing positivity for IFN-γ-Th1 either for conidia either for a water cellular extract of Aspergillus, while resulted completely negative in the six previous patients. ELISPOT has been demonstrated the only proof of IA in our patient, in whom the diagnosis has been reached only after surgical

procedure, being all the available tests, including galactomannan, repeatedly negative. ELISPOT has provided the first description of immune response to an IA during the course of the disease, *in vivo*, showing: high levels of IL-10-Th2 at onset and during progression, growing levels of IFN-γ-T h1 during regression/resolution, but counterbalanced by sustained level of IL-10-Th2 to avoid an excessive inflammatory reaction; extending previous finding. Thus ELISPOT may represent a step forward in understanding the paradigm of the IFN-γ-T h1/ IL-10-Th2 dynamic skewing at each time point of IA, with possible consequences in designing therapeutic strategies in high risk patients. Our positive finding demonstrates the potential of ELISPOT in the diagnosis of IA and combined with the negative results in control patients with pneumonia of non fungal etiology, may spur further studies to validate the use of ELISPOT as new tool for the early diagnosis of IA in all high risk patients, including those receiving and alloBMT or a solid organ transplant.

PO-104

ID-ARA-C, IDARUBICINE AND GO AS SALVAGE TREATMENT IN ADVANCED AML PATIENTS

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Currently the treatment of relapsed and refractory acute myeloid leukaemia patients remains a major problem. Whenever second CR is attained, the median duration of the second relapse-free interval (RFI) is generally considerably shorter than that of the first, at least in patients not eligible for transplant procedures. The major aim of our study was to evaluate the feasibility and efficacy, in term of second CR rate and survival, of a salvage chemotherapy consisting of: Ara-C 1 gr/m^2/day, in 4 hours (days 1,5); Idarubicine 8 mg/m^2/day, in 1 hour (days 1,3,5); GO 3 mg/m^2/day (day 6); Peg-G-CSF 6 mg/day (day 8). The patients achieving CR were planned to receive a consolidation course with the same drug and schedule followed by allogenic transplantation. In our institute 11 patients were treated according to this protocol; 9 of the 11 patients were in first relapse, while 2 were refractory to first line chemotherapy. The main characteristics are shown in the Table.

Table 1.

		Patients characteristics					
Patient	*Age Sex*	*Cytogenetic*	*Molecular Biology*	*CR1 duration*	*BMT in CR1*	*MIF CD33/blast*	*n°mol CD33/blast*
1	26/F	47XX,t(12;17),+der17	neg	REF	no	376	10935
2	60/F	normal	neg	4 months	no	825	24870
3	56/M	normal	FLT3-ITD +	3 months	no	886	22109
4	61/F	normal	neg	9 months	autologous	384	10573
5	46/M	normal	FLT3-ITD +	REF	no	773	22824
6	25/M	unknown	unknown	2 months	no	55	1821
7	58/F	normal	FLT3-ITD +	1 months	no	552	17775
8	52/M	inv 16	CBFβ-MYH11,tipoA	26 months	autologous	48	1170
9	50/M	normal	neg	53 months	no	33	1426
10	27/F	t(11;19)(q23;p11)	neg	22 months	no	658	13560
11	28/F	t(9;11)	neg	6 months	sibling	110	3344

**MIF = Mean Intensity Fluorescence*

Overall response rate was 81% (6 CR, 1 CRp and 2 PR). 2 patients, refractory to first line chemotherapy, did not show any response and died. Extrahaematological toxicity was mild: only one patient presented grade 3 oral mucositis. No patients presented alopecia. Recovery was evaluable in 8/11 patients. The median time required to attain an absolute neutrophil count in excess of 0.5×10^9/L was 19 days (range 17-26). A sustained platelet count exceeding 50×10^9/L was reached after a median of 31 days (range 19-50 days). Nine patients had fever (5 documented infections, 2 FUO and 2 pneumonia). Empirical antifungal therapy was administered in two patients because of persistence of fever. Out of the 7 patients in second CR, 1 relapsed after consolidation and died, 1 underwent sibling transplantation and is alive in second CR 1 month after BMT, 1 patient is receiving consolidation course, 3 are receiving allogenic transplantation after a follow-up of 3, 2 and 1 month from second CR, 1 patient, relapsed after sibling transplantation in first CR, underwent donor lymphocyte infusion after one month from second CR. Two patients who achieved PR relapsed, rapidly, after consolidation therapy and died. Although the relatively small cohort of patients, the regimen utilized seems promising and feasible, allowing to proceed to transplantation in the majority of them. Obviously a longer follow-up and a prospective multicenter study is necessary in order to confirm our data.

PO-105

ETIOLOGICAL AGENTS AND RISK FACTORS FOR INFECTIOUS COMPLICATIONS IN HAEMATOLOGICAL PATIENTS WITH CENTRAL VENOUS CATHETERS: A SINGLE CENTER PROSPECTIVE ANALYSIS

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Background. Patients (pts) with haematological malignancies who need central venous catheters (CVCs) are at risk of developing several complications of which catheter-related infections are prevalent and often life-threatening. *Aims.* The purpose of this single-centre, prospective study was to evaluate frequency, epidemiology, risk factors and outcome of catheter-related infectious complications in this subgroup of patients. *Methods.* Between September 2002 and December 2004, a total of 388 procedures were performed in 279 patients(137 male and 142 female, mean age 49.7 yrs, range 17-75). Hematological diagnosis comprised: acute myeloid leukaemia (120 pts, 43%), acute lymphoid leukaemia (24 pts, 8.6%), Hodgkin and non-Hodgkin lymphomas (70 pts, 25.1%), multiple myeloma (44 pts, 15.8%), others (21 pts, 7.5%). Reasons for CVC placement differed among pts subgroups: chemotherapy administration and supportive therapy during aplasia in acute leukaemia pts; peripheral blood stem cell harvest in lymphoma and myeloma pts. Growth of the same microrganism both in catheter and peripheral vein blood cultures, with no other documented source of infection other than the catheter itself, defined a catheter-related bloodstream infection (CR-BI). CVC exit point cutaneous infections were also recorded. *Results.* CR-BI complicated 49 of 388 procedures, 35 acute leukaemia pts, 8 lymphoma/myeloma pts, with a frequency of 6.7 events per 1,000 catheter days; CVC exit point infections were documented in 19 pts, 14 acute leukaemia pts and 2 lymphoma/myeloma pts, with a frequency of 2.6 events per 1,000 catheter days. Overall mean catheterization duration was 18.8 days; overall mean duration of severe neutropenia (absolute neutrophil count <0.1×10^9/L) during catheter *in situ* maintenance was 4.7 days; mean duration of ANC <0.5×10^9/L was 7.4 days. Both neutropenia and catheter permanence were most pronounced in acute leukaemia pts, as expected (mean duration of catheterization: 23.8 days; mean neutropenia with ANC <0.5×10^9/L: 11.3 days; mean neutropenia with ANC <0.1×10^9/L: 7.3 days). In 34 cases a Gram-positive CR-BI was documented, with S. epidermidis and streptococci strains being the most frequently isolated germs, 20 and 6 cases respectively. The remaining were Gram-negative CR-BI, most of which caused by E.coli (4 cases), P. aeruginosa (3 cases) and Enterobacter spp (3 cases). No fungal CR-BIs were diagnosed. During the study period no pt died due to CR-BI; two pts died of disease progression. Catheter removal because of severe infectious complications was necessary in 14 of 388 cases (3.61%). At univariate analysis, risk factors for CR-BI development were: number of catheter days (p<0.0001), chemotherapy dose (high vs. standard dose; p<0.015), duration of neutropenia (p<0.001) and thrombocytopenia (p<0.001). At multivariate analysis, only catheter days and duration of neutropenia appeared significant risk factors for CR-BI. *Conclusions.* On the whole, in our study population frequency of catheter-related infectious complications was modest: CR-BI occurred in only 49 of 388 procedures (12.6%). Risk factors for CR-BI are strictly related to the clinical characteristics of pts treated with chemotherapy for their hematological malignancy, with neutropenia and thrombocytopenia being two relevant risk factors at univariate analysis. Duration of catheterization in this setting of increased susceptibility to infections obviously plays an important role also. It follows that acute leukaemia pts are particularly prone to catheter-related infections (35 of 49 cases), especially when high-dose chemotherapy for induction and salvage therapy is administered.

Gene Therapy

PO-106

IMMUNOPHENOTYPIC HETEROGENEITY OF BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS (BM-MSCS) FROM PATIENTS WITH HEMATOLOGICAL DISORDERS: CORRELATION WITH CULTURE CONDITIONS AND BONE MARROW MICROENVIRONMENT

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The assessment of the immunophenotypic profile of ex-vivo expanded mesenchymal stromal cells (MSCs) has been confined to single or dual staining analysis. Moreover, most of the reports dealt with MSCs from normal subjects (NS-MSCs), while only a few studies were related to MSCs obtained from the BM of patients with hematological malignancies (HM-MSCs). Long-term cultured BM-derived MSCs from 8 NS and 43 patients with HM were studied by 4-color cytofluorimetric analysis using a wide panel of monoclonal antibodies in different combination to uncover possible immunophenotypic differences. MSCs were analysed using a gating strategy based on the exclusion of CD45+/ 7-AAD+ (7-aminoactinomycin D) events. Our data demonstrated that BM- MSCs from HM showed alterations in the expression of CD105, CD90, CD184, and HLA-DR molecules. The decrease in the percentage of CD105 and CD90+ MSCs correlated with an increased BM angiogenesis. Interestingly, skin fibroblasts (SF) resulted negative for CD106. Several immunological markers used to identify MSCs were also expressed by CD45+ leukemic blasts. The comparative analysis of 4 different *in vitro* culture conditions, allowed us to demonstrate that the most commonly used *in vitro* supplements (bFGF,VEGF,EGF) greatly stimulated MSC proliferation, but unexpectedly downregulated CD90 (Thy-1) and, in a lesser extent, CD146 expression on HM-MSC, and CD105 in SF; in contrast this culture condition up-regulated CD44, CD10, CD271 (LNGFR), and HLA-DR molecules in some patients with lymphoproliferative disorders. In conclusion, this paper provides evidence that multiparameter flow cytometry is essential for the establishment of a standardized protocol aiming at the identification of various MSC subsets and of aberrant phenotypes, and for a careful assessment of the effects of microenvironmental conditions on MSCs function.

PO-107

AMNIOTIC-FLUID MESENCHYMAL STEM CELLS AS POTENTIAL FOR THERAPEUTIC TRANSPLANTATION?

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Mesenchymal stem cells (MSCs) are multipotent precursors capable of differentiating into bone and cartilage and adipose tissue. Many genetic disorders cause a defective production of extra-cellular matrix (ECM) and they are responsible for irreversible damages to the fetus. One of these disease is the Osteogenesis Imperfecta (O.I.), a genetic disorder caused by defects in Collagen type I (Col I). Col I is the major structural protein of the bone extracellular matrix. Recently has been reported that the amniotic fluid is a valid source of MSC cells. These cells could be a valid target for the genetic correction of the molecular defect. The aim of this study was to isolate MSCs from amniotic fluid obtained by second-trimester amniocentesis and to differentiate them into osteoblasts. This type of cells were successfully isolated, cultured and enriched without interfering with the routine process of fetal kariotyping because they were obtained from the supernatant of first week media change. Amniotic-fluid MSCs (AFMSCs) were cultured under specific conditions for 8 weeks and analysed by flow cytometry and quantitative real time PCR to asses the presence and the expression levels of specific markers. AFMSCs were tested after 6, 7 and 8 culture weeks. Flow cytometry analyses showed that they were positive for SH3, SH4, CD90, CD44 and CD29, low positive for CD105, but negative for CD45, CD34, CD25, CD31, and HLA-DR. The gene expression levels of the markers investigated agreed with the flow cytometry data. Phenotypical characterization by flow cytometry revealed a peak of expression of the specific markers for the MSCs in 8 weeks. These cells cultured in a mesenchymal specific media for 8 week, were induced to differentiate into osteoblasts. After 3 weeks AFMSCs derived-osteoblasts were valutated for their morphology and capacity to deposit calcium mineral in the extracellular matrix using Alizarin red-S. *Conclusions.* We demonstrate with the immunoistochemestry and molecular techniques the presence of human multi-potent MSCs in the second-trimester amniotic fluid. These cells could have a great potential for both cells therapy and for gene therapy. Indeed most of them origin from the skin and from the urinary tract of the fetus. These cells be considered the best candidate to support our intrauterine fetal tissue engineering project for their availability and because they have been demonstrated to be valid multipotent precursors for cartilage, adipose tissue and bone.

PO-108

ADULT HUMAN AND MOUSE SPLEEN AND THYMUS: ALTERNATIVE SOURCES OF MESENCHYMAL STEM CELLS WITH THE SAME MULTILINEAGE DIFFERENTIATION POTENTIAL AND IMMUNOREGULATORY PROPERTIES OF THOSE FROM BONE MARROW

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Spleen and thymus are lymphoid organs with a pivotal role in the immune system development and function, and therefore very sensitive to the effect of immunoregulatory cells, but also with a well-structured stromal architecture of uncertain function and origin. We asked whether spleen and thymus have a reservoire of mesenchymal stem cells (MSCs) with multilineage potential and whether the onset of immune responses may be affected by these cells in normal and pathological conditions, such as tumour growth. For these purposes: i. we have applied the procedures currently used to obtain MSCs from bone marrow, to human and mouse spleen- and thymus-derived cell suspensions; ii. we have assessed *in vitro* immunoregulatory potential with different immune effector cells; iii. we have used a mouse model to clarify the kinetics of *in vivo* inhibitory effect of MSCs of different origin during the development of an anti-cancer (Sp6 myeloma cells) immune response. We have found that MSCs can be achieved not only from bone marrow, but also from spleen and thymus of both human and mouse origin. *In vitro*, human and mouse spleen- and thymus-derived MSCs have immunophenotypic characteristics and differentiation potential completely comparable to bone marrow MSCs. As regards the regulatory effect on activated lymphocytes, all these MSCs can inhibit the immune responses with the same efficiency of bone marrow MSCs. *In vivo*, mouse MSCs from bone marrow, spleen and thymus, if injected subcutaneously together with tumour cells during immunization, can equally prevent memory immune response against Sp6 tumour cell line, thus leading to tumour growth in mice normally resistant. Our data suggest that not only do the spleen and thymus have a stem cell reservoire to build up their stromal architecture and with multilineage differentiation potential, but also originate microenviromental immunoregulatory cells that may have a role in lymphocyte priming and activation against antigens.

PO-109

DENDRITIC CELLS INCUBATION WITH ZOLEDRONATE IMPROVES THE ABILITY TO MODULATE INNATE AND ADAPTIVE IMMUNE RESPONSES

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Zoledronic acid (Zol) is an aminobisphosphonate commonly used in the treatment of bone lesions in hematological and solid cancers. Zol shows immunomodulatory properties, i.e. the activation of γ9δ2 T cells (γδ T), mediated by monocytes. γδ T constitute a small subset of peripheral blood lymphocytes involved in the response to microbial antigens and in the immunosurveillance against tumors. We investigated the effect of Zol on dendritic cells (DC), which are professional antigen-presenting cells interacting with both innate and adaptive immunity effectors. METHODS: immature DC (iDC) were generated from peripheral blood monocytes, immunomagnetically selected (purity >95%), and cultured with GM-CSF + IL-4. Mature DC (mDC) were obtained with

24h exposure to LPS or TNF-α + IL-1β (maturation cytokine cocktail, MCC). After 24h exposure to Zol, DC were incubated for 7 days with monocyte-depleted PBMC and than washed: γδ T were identified by flowcytometry as CD3+/γδ-TCR+ cells. Antigen (Ag)-specific response was induced by 2-rounds stimulation of T cells with autologous mDC loaded with HLA-A2-restricted flu-peptide. T cells cytotoxic effect against peptide-pulsed HLA-A2+ T2 cell line was measured cytofluorimetrically by CFSE/propidium staining. RESULTS: Short-term Zol-treatment did not increase the percentage of apoptotic DC (<8%). Zol-treated iDC maintained their ability to endocyte soluble antigens (43% FITC-dextran+ iDC). On mDC, Zol-treatment enhanced the expression of maturation molecules (CD80 fluorescence intensity: no Zol 263±57 vs. Zol 349±85, n=8, p<0,03), did not affect IL-12 production, and modulated the secretion of proinflammatory cytokines (IL-6: LPS 3.245 vs. LPS+Zol 6.614 pg/mL; MCC 6.647 vs. MCC+Zol 1.364 pg/mL). Zol-treated DC were able to induce γδ T proliferation, more efficiently than monocytes (iDC 233.578±84.044 cells/well, mDC 250.424±92.035 cells/well, monocytes 104.722 ± 37.729 cells/well). Finally, Zol-treatment of mDC did not hamper their priming ability on the αβ T cell population: peptide-pulsed DC simultaneously with Zol-treatment elicited the Ag-specific cytotoxic effect of αβ T cells against flu peptide-loaded T2, as well as the γδ T innate response. CONCLUSION: Zol-treatment of *in vitro* generated DC can be exploited as immunotherapeutical tool to modulate the interaction between innate and adaptive immune response.

PO-110

SELECTIVE APOPTOSIS OF MONOCYTES AND MONOCYTE-DERIVED DENDRITIC CELLS INDUCED BY BORTEZOMIB (VELCADE)

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Bortezomib (Velcade), a proteasome inhibitor, has shown immunosuppressive activity in several *in vivo* models of inflammatory diseases, including GVHD and autoimmune diseases. *In vitro*, bortezomib affects the survival and function of dendritic cells (DC) and certain lymphocyte subsets. Since monocytes are a major circulating source of DC and their role seems critical in inflammatory disorders, such as systemic lupus erythematosus (SLE) and graft-versus-host disease (GVHD), in this study we evaluated the effects of bortezomib on the survival of monocytes, compared to other peripheral blood (PB) leukocyte subsets. Unfractionated PB mononuclear cells (PBMC) were cultured in medium for 24 hrs with different concentrations of bortezomib (1-100 ng/mL), which are comparable to those utilized in clinical trials. After culture the cells were analyzed by flow cytometry. Compared to control cultures that included 10+3% $CD14^+$ monocytes, cultures with bortezomib showed a significant dose-dependent reduction (p=0.008, n=3) of monocytes (1.9+1.6% at 5 ng/mL, 0.3+0.1% at 10 ng/mL, <0.1% at 50 ng/mL and 100 ng/mL), with <1% $CD14^+$ cells at doses of bortezomib > 5 ng/mL. Among $CD14^+$ cells, CD16- and CD16+ monocyte subsets were equally depleted. Similarly, circulating DC, identified as HLA-DR+ lineage negative cells, were not detectable (<0.01%) after culture with bortezomib > 5 ng/mL, as compared to 0.2-0.4% in control cultures. When immunomagnetically purified $CD14^+$ monocytes were cultured with GM-CSF and IL-4, the addition of bortezomib significantly decreased the number of monocytes within 24 hrs of culture (p=0.004) (n=6). Monocyte loss was due to apoptosis, demonstrated by annexin V-mediated detection of phosphatydilserine on cell membrane, which increased to 60+25% in cultures with bortezomib at 1 ng/mL and 88+6% at 10 ng/mL, as compared to 46+16% in control cultures (p=0.0002, n=6), after 24 hrs culture (approximate effective dose 50%, ED50, 1-10 ng/mL). In addition, both immature DC, derived from monocytes after culture for 6 days with GM-CSF and IL-4, and mature DC, obtained from immature DC after 24-48 hrs culture with LPS, underwent apoptosis following exposure to bortezomib for 24 hrs (ED50 of 1-10 ng/mL and 10-50 ng/ml, respectively). Kinetics experiments were performed by measuring the induction of apoptosis in monocytes after exposure to bortezomib for 4, 8, 12, 16 and 24 hrs at 10 ng/mL. Annexin V staining was stable for up to 12 hrs (21+1%), whereas it increased at 16 hrs (41+5%) through 24 hrs (83+3%) (n=2). However, short time (<12 hrs) incubation with bortezomib irreversibly committed monocytes to undergo apoptosis following extensive washing and culture in the presence of GM-CSF and IL-4 (43+15% annexin V+ cells at 24 hrs, 74+14% at 72 hrs and 68+30% at 6 days as compared to 22-28% in control cultures). The selectivity of bortezomib was further confirmed by observing no effect on purified $CD19^+$ B and CD3+ T lymphocytes (ED50: > 100 ng/mL for both cell populations in culture w/wo mitogens). Furthermore, bortezomib did not induce apoptosis in purified CD34+ progenitor cells cultured w/wo IL-3 (ED50 >100 ng/mL). These results demonstrate a selective inhibitory effect of bortezomib on professional antigen-presenting cells, such as monocytes and dendritic cells. Based on these findings, future studies could be hypothesized to test the clinical effect of bortezomib in monocytic/hystiocytic diseases, or in inflammatory disorders such as SLE or acute GVHD.

PO-111

INDUCTION OF NEURAL DIFFERENTIATION IN HUMAN MESENCHYMAL STEM CELLS DERIVED FROM BONE MARROW, FAT, SPLEEN AND THYMUS

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The great majority of studies regarding neural differentiation potential of mesenchymal stem cells (MSCs) have been focused on the capacity of adult bone marrow-derived MSCs to differentiate into neurons and/or glial cells of the central nervous system. A more limited number of studies concerns adult MSCs derived from tissues other than bone marrow: cells with morphological and biological features of neural cells have been obtained from fat-derived MSCs, but the neural differentiation potential of other adult tissues is still unknown. Aim of our study was to evaluate and compare the neural differentiation potential of adult human MSCs obtained from different tissues (bone marrow, fat, thymus, and spleen), using previously published protocols of chemical induction, with particular attention to the duration and selectivity of such a process; in addition, we assessed the effects of MSC-Schwann cell co-cultures on the process of neural differentiation. Under the effect of neural differentiation medium, most MSCs from bone marrow, fat, spleen and thymus acquired morphological (as assessed by light and electron microscopy) and phenotypic changes suggestive of cells of astrocytic/neuronal and oligodendroglial lineages. The analysis of neural markers by immunocytochemistry showed that most *differentiated* MSCs displayed a general up-regulation of neural molecules. The process was transient and reversible, as MSCs recovered basal morphology and phenotype, as well as their multi-lineage differentiation potential. By contrast, co-cultures of MSCs of different origin with Schwann cells induced long-lasting and selective glial differentiation, i.e. the expression of Schwann cell-associated myelin proteins for up to 12 days. The present results show that a MSC reservoir is present in tissues other than bone marrow and fat, and that MSCs of different origin have similar neural differentiation potential. This evidence provides new insights into tissue plasticity and may have important implications for future therapeutic interventions in chronic neuropathies.

PO-112

DEVELOPMENT OF AN ANTILEUKEMIC VACCINE APPROACH BASED ON THE WT1 FULL LENGTH PROTEIN

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Background. The Wilms' tumor gene is overexpressed in many types of haematological malignancies including acute myeloid leukaemia (AML), acute lymphoid leukaemia (ALL), chronic myeloid leukaemia (CML) and Ph negative myeloproliferative disorders. WT1 fulfils, from the immunological point of view, the characteristics of a the tumor-associated antigen: it is expressed at high levels in blast cells but not in normal tissues and it is involved in the maintenance of malignant phenotype.Many clinical trials using MHC class I-restricted WT1 peptide, have

been recently performed in patients affected by AML, MDS, lung and breast cancer with satisfactory clinical results and without relevant toxicity. The aim of the study was to analyze the natural immunity against WT1 in different types of haematological malignancies. Moreover in order to circumvent some limits of peptides vaccination, such as the HLA restriction, we set up in the mouse model a vaccination approach using the WT1 full length protein and we test the safety and efficacy of this vaccine. Materials and methods: Using a dot blot technique, we analyzed for the presence of WT1 specific antibodies 92 patients including: 18 AML, 20 myelodysplastic syndromes (MDS), 22 Multiple myeloma (MM), 14 CML, 12 idiopatic myelofibrosis (IM), 6 Chronic Myelomonocitic Leukaemia (CMML) and 30 normal subjects as control. Moreover, we set up a vaccination approach in the mouse model (C57BL/6) based on the administration of the WT1 full length protein. Complete coding sequence was cloned in a pGEX bacterial expression vector for the production of the fusion protein GST-WT1 that was subsequently purified by glutathione conjugated beads. Ten C57BL7/6 mice were immunized with 50 microg of purified fusion protein every 15 days, for a total of 3 immunizations and 10 mice were used as control. 2 weeks after the last administration, five vaccinated mice and five controls have been injected with 200.000 TRAMP-C2 cells. Five mice for each group were sacrificed and lymphocytes, cultured in presence of interleukin-2 to perform a cytotoxicity assay. Sections of different tissues were prepared for histochemical analysis to exclude a toxic effect. Results: Dot blot analysis allowed to demonstrate a significant higher level of antibody against WT1 in patients as compared to normal controls: the higher levels were detected in CML, ALL and IM but significant levels were observed also in MDS, AML, CMML and MM. Finally, the injection of WT1 protein did not result in any kind of organ toxicity. Importantly, immunized mice showed high level of CTL and antibody against WT1 and this resulted in a significant reduction of the tumour burden. *Conclusions.* Data obtained from this study clearly demonstrated that WT1 protein is able to elicit an immune response. Although further studies are required to compare the efficacy of this approach to the peptide based vaccine, WT1 full length protein could represent an optimum vaccine allowing to overcome the HLA restriction limit of the peptide approach.

PO-113

EFFECTIVE ANTI-TUMOR IMMUNOMODULATORY PROPERTIES OF ZOLEDRONIC ACID IN BALB/C MICE TRANSGENIC FOR THE HER-2/NEU ONCOGENE

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Zoledronic acids (Zol) is the most potent aminobisphosphonates clinically available.[1] Preclinical *in vivo* data suggest that it modulates the development of bone disease, decreases tumor burden[2] with a direct anti-cancer activity,[3-5] and reduces the migration and the metastatic invasion of cancer cells.[6] Zol efficacy in anti-cancer adjuvant therapy also rests on its anti-angiogenic properties[2,7] and ability to expand γ/δ T cells, both *in vitro* and *in vivo*.[8-10] The published data thus far available concern murine models of transplanted tumors.[2,4] However, a recent report shows that Zol impairs tumor-associated angiogenesis in a transgenic model of cervical carcinoma.[7] Present work was made to assess Zol ability to impair spontaneous carcinogenesis in a transgenic mouse model. Virgin BALB/c female mice transgenic for the activated rat Her-2/neu oncogene (BALB-neuT mice) provide one of the most aggressive and consistent model of autochthonous mammary carcinogenesis.[11] The step-wise pattern of mammary tumor progression in these mice closely mimics that of breast carcinoma in women, thus providing a realistic model for assessing the efficacy of Zol as an anti-tumor agent. The inexorability of the development of a palpable mammary tumor by week 15 by all BALB-neuT mice allows an assessment of the protection afforded as the extension of the disease-free survival and the percentage of tumor-free mice as time progresses. Moreover, as a tumor is palpable in all ten mammary glands around week 33, the tumor multiplicity can also be assessed and the increase in the size of each lesion can be measured.[12-14] Previous data have shown that the early stages of this aggressive neoplastic progression can be hampered by the stimulation of the immune system, showing that cure of mammary carcinomas in Her-2 transgenic mice could be obtained by an appropriate stimulation of both innate (neoadjuvant interleukin-12,[11] α-galactosylceramide[15] and adaptive (DNA vaccine electroporation[16,17] immunity. This model therefore have shown to provide an ideal frame to explore the immunomodulant properties of various compounds. To asses Zol anti-tumor and immunomodulant properties, BALB-neuT mice were treated with 16 administration of 100 μg/Kg of Zol divided into four courses of a single weekly injections for four weeks followed by a three weeks rest. Zol administration was started when mice were 7 weeks old and therefore when all the 10 mammary glands display a widespread atypical hyperplasia. Zol was administered intravenously (i.v.) or into the mammary pad (i.mam.). Mice were evaluated for: 1) tumor onset, 2) tumor multiplicity and 3) overall survival. Data obtained from these first experiments have shown that a similar significant tumor growth impairment was evident in mice receiving Zol administered i.v. or i.mam. In both cases, Zol was as effective as IL-12 in impairing the mammary carcinogenesis and the tumor progression, thus suggesting a possible immuno-mediated mechanism in addition to its direct anti-tumor and anti-angiogenic effects. To investigate the mechanistic basis of Zol anti-tumor properties Zol-treated mice were sacrificed at various time-points after Zol administration and its *in vivo* immunomodulatory properties were evaluated. Preliminary results have shown Zol capacity to induce a significant increase in the percentage of γδ T cells in the spleen and in the lymphonode of Zol-treated mice. Interestingly, peripheral blood γδ T cells were not expanded, thus indicating a preferential homing of these cells in the secondary lymphoid organs. Similarly, Zol treatment induced a significant increase in the expression of costimulatory molecules on the surface of B cells and dendritic cells (DCs) isolated from mice spleen and the lymphonodes, but not from peripheral blood. Even more importantly, data recently obtained in IFNγ knocked out -NeuT mice have shown that Zol capacity to delay tumor onset and growth and to improve NeuT-mice survival is strictly dependent on the presence of IFNγ, which is a well known mediator of innate and adaptive immune responses. All together, these data show for the first time that Zol anti-tumor functions at least partially relies on its immunomodulant properties, thus providing the rationale to better explore and exploit Zol immunoadjuvant properties in the context of antitumor vaccination.

References

1. Rosen LS, Gordon D, Kaminski M, Howell A, Belch A, Mackey J, et al. Long-term efficacy and safety of zoledronic acid compared with pamidronate disodium in the treatment of skeletal complications in patients with advanced multiple myeloma or breast carcinoma: a randomized, double-blind, multicenter, comparative trial. Cancer 2003, 98:1735-44.
2. Croucher PI, De Henrik L, Perry MJ, Hijzen A, Shipman CM, Lippitt J, et al. Zoledronic acid treatment of 5T2MM-bearing mice inhibits the development of myeoma bone disease: evidence for decreased osteolisys, tumor burden and angiogenesis, and increased surival. J Bone Miner Res 2003; 18 :482-492
3. Yaccoby S, Pearse RN, Jhonson CL,; Barlogie B, Choi Y, Epstein J. Myeloma ineracts with the bone marrow microenvironment to induce osteoclastogenesis and is dependent on osteoclast activity. Br j of Heamatol 2002; 116:278-90.
4. Kuroda J, Kimura S, Segawa H, Kobayashi Y, Yoshikawa T, Urasaki Y, et al. The third-generation bisphosphonate zoledronate synergistically augments the anti-Ph+ leukemia activity of imatinib mesylate. Blood 2003, 102:2229-35.
5. Kabelitz D, Wesch D, Pitters E, Zoller M. Characterization of tumor reactivity of human V γ 9V δ 2 γδ T cells in vitro and in SCID mice in vivo. J Immunol 2004, 173:6767-76.
6. Hiraga T, Williams PJ, Ueda A, Tamura D, Yoneda T. Zoledronic acid inhibits visceral metastases in the 4T1/luc mouse breast cancer model. Clin Cancer Res. 2004, 10:4559-67.
7. Giraudo E, Inoue M, Hanahan D. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. J Clin Invest. 2004, 114:623-33.
8. Kunzmann V, Bauer E, Feurle J, Weissinger F, Tony HP, Wilhelm M. Stimulation of gd T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. Blood 2000, 96:384-92
9. Dieli F, Gebbia N, Poccia F, Caccamo N, Montesano C, Fulfaro F, et al. Induction of γδ T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients in vivo. Blood 2003, 102:2310-1.
10. Mariani S, Muraro M, Pantaleoni F, Fiore F, Nuschak B, Peola S, et al. Effector γδ T cells and tumor cells as immune targets of zoledronic acid in multiple myeloma. Leukemia 2005, 19:664-70.

11. Boggio K, Nicoletti G, Di Carlo E, Cavallo F, Landuzzi L, Melani C et al. Interleukin-12 mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. J Exp Med 1998;188: 589-96.
12. Di Carlo E, Diodoro MG, Boggio K, Modesti A, Modesti M, Nanni P et al. Analysis of mammary carcinoma onset and progression in Her-2/neu oncogene transgenic mice reveals a lobular origin. Lab Invest 1999; 79:1261-9.
13. Pannellini T, Forni G, Musiani P. Immunobiology of Her-2/neu transgenic mice. Breast Dis 2004; 20:33-42.
14. Astolfi A, Landuzzi L, Nicoletti G, De Giovanni C, Croci S, Paladini A, et al. Gene expression analysis of immune-mediated arrest of tumorigenesis in a transgenic mouse model of HER-2/neu-positive basal-like mammary carcinoma. Am J Pathol 2005; 166:1205-16.
15. Hayakawa Y, Rovero S, Forni G, Smyth M. Galactosylceramide (KRN7000) suppression of chemical- and oncogene-dependent carcinogenesis. Proc Natl Acad Sci USA 2003; 100:9464-9.
16. Quaglino E, Rolla S, Iezzi M, Spadaro M, Musiani P, De Giovanni C et al. Concordant morphological and gene expression data shows that vaccination halts Her-2/neu preneoplastic lesions. J Clin Invest 2004; 113:709-17.
17. Quaglino E, Iezzi M, Mastini C, Amici A, Pericle F, Di Carlo E et al. Electroporated DNA vaccine clears away multifocal mammary carcinomas in Her-2/neu transgenic mice. Cancer Res 2004; 64:2858-64.

PO-114

TRANSGENIC EXPRESSION OF IL-15 IN ANTIGEN SPECIFIC CYTOTOXIC T CELLS (CTLS) SELECTIVELY ENHANCES THE EXPANSION OF TRANSGENIC CELLS

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One limitation of the adoptive transfer of antigen specific CTLs appears to be the reduced capacity of these cells to expand *in vivo*. Systemic administration of IL-2 may be tonic and induces expansion of unwanted cells including T-reg cells. Transgenic expression of lymphokines is a potential method to overcome these problems, allowing CTLs to sustain their own expansion. Both IL-2 and IL-15 sustain the expansion of T cells, but only IL-15 has the advantage of increasing the memory T cell pool and has limited effects on T-reg cells. We have compared the effects of the transgene expression of IL-2 and IL-15 in our model of EBV-specific CTLs. We cloned full length hIL-2 and hIL-15 into the SFG retroviral vector. To provide a selectable marker to sort and track these cells for a potential clinical use, we cloned a truncated form of the human CD34 molecule within the cassette using the 2A cleavable sequence. A suicide gene based on inducible caspase 9 molecule was also included to provide a mechanism of selective elimination of transgenic T cells in case of uncontrolled proliferation. The growth kinetics of EBV specific CTLs expressing IL-2 or IL-15 in combination with the expression of the iCaspase9 suicide gene were compared. We observed in 4 different donors a significant expansion of CTLs expressing IL-2 (43±6 fold expansion) or IL-15 (27±7 fold expansion) after 21 days of culture compared to control CTLs maintained in culture without any addition of exogenous cytokines (0.1±0.5 fold expansion). Transgenic CTLs maintained the same phenotype of control T cells (CD3+/CD8+ >90%) and maintained antigen specificity, as assessed by tetramer staining and 51Cr release assay. Interestingly, we found that the percentage of CD34+ cells increased overtime for IL-15 transgenic CTLs (from 30±9% to 68±13% by day 21 of culture), while this percentage remained unchanged for IL-2 transgenic CTLs (from 30±6% to 36±7%) suggesting that IL-15 induced CTL expansion preferentially through an autocrine mechanism. To evaluate the functionality of the suicide gene, CTLs were incubated with the dimerization drug (CID AP20187) (20 nM). Twenty-four hours later, less than 3% CD34+ cells were detectable in the culture and production of cytokine was below the detection limit. Preliminary experiments *in vivo* using a SCID mouse model engrafted with LCL/EBV-CTL and an *in vivo* bioluminescent system (Xenogen-IVIS) to track the CTLs, showed that transgenic cytokine expression improves expansion and persistence of the transgenic CTL compared to control cells. We are currently evaluating whether these modifications improve the anti-tumor effect of EBV-CTLs *in vivo* and whether the suicide gene allow a long-term control of transgenic CTL making this approach suitable for safe clinical application.

PO-115

ANTI-IDIOTYPE DNA VACCINATION BY GENE GUN IN THE MURINE BCL1 LYMPHOMA MODEL

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Most B- and T-cell lymphomas retain expression of the B-cell receptor (BcR) and the T-cell receptor (TcR), respectively. Thanks to the clonal origin of both the immune system and the neoplasia, these antigen receptors can act as tumour-specific antigens (TSA) able to stimulate production of protective antibody as well as $CD4^+$ and $CD8^+$ T lymphocytes. Anti-idiotype DNA vaccination can be made more immunogenic using plasmids encoding small immunoproteins (SIP), consisting of a single-chain variable fragment (scFv) derived from the tumor idiotype and fused in-frame with a xenogeneic immunoglobulin constant region (in our model the human IgG1 CH3 domain) to allow dimerization, with or without the addition of a plasmids encoding the adjuvant GM-CSF (pGM-CSF). Recent studies have shown that one of the most immunogenic routes of administration for plasmid DNA is the bioballistic one (so called *gene gun*), which transfects only epidermal and dermal cells (exploiting the reactogenicity of this immunological barrier) and requires only a few micrograms of DNA, minimizing chromosomal integration risks. In the murine BCL1 lymphoma model, this approach has already proved to be partially effective at inducing protective antibodies in 40-50% of syngeneic BALB/c mice despite the occurrence of remaining dormant circulating lymphoma cells. Since topical imiquimod (a synthetic compound already approved in humans for dermatological uses) has been shown to induce T helper 1 (Th1) polarization of $CD4^+$ T lymphocytes by binding TLR7 on plasmacytoid dendritic cells, this work investigated its use as an adjuvant to induce activation of cell-mediated antitumor immunity in the anti-BCL1 idiotype vaccination model and to assess if this correlated with improved overall survival. The results presented here discourage the use of pGM-CSF and imiquimod either alone or in combination, at least with our temporal and dosing schedules : these findings have important implications for the design of future clinical trials.

Chronic Myeloid Leukemia I

PO-116

THE CATALYTIC ACTIVITY OF BCR-ABL MODULATES SURVIVIN EXPRESSION IN CHRONIC MYELOGENOUS LEUKEMIA CELL LINES

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Chronic Myelogenous Leukemia (CML) is a myeloproliferative disorder caused by a chromosomal translocation juxtaposing the first exons (13 or 14) of the BCR gene to the last 10 exons of the ABL gene. The chimeric oncogene BCR-ABL encodes for a cytoplasmic protein with a constitutive tyrosine kinase activity which lead to the suppression of many apoptotic pathways. To better define the mechanisms responsible for the chemoresistance in CML cells, we investigated the expression and function of Survivin, an Inhibitor of Apoptosis Protein (IAP), in such a system and his relation with BCR-ABL. Using the 32D murine myeloid cells retrovirally transfected with BCR-ABL or gfp (as a control) we found that expression of BCR-ABL increased Survivin levels. This increase was dependent on BCR-ABL kinase activity since treatment for 48 hrs with Imatinib Mesylate (IM), a semi-specific ABL kinase inhibitor, has led to the down-regulation of Survivin. To further confirm that BCR-ABL kinase activity was responsible for the increased expression of Survivin, we repeated these experiments in four human CML cell lines and in HL60 cells (BCR-ABL negative cells) used as a control. The cells were grown for 48 hrs in the absence or presence of IM. Anti-Survivin immunoblots showed a reduction in the expression of Survivin in the CML lines but not in the HL60 cells. We hypothesized that the up-regulation of this IAP required a BCR-ABL downstream pathway. However, when we treated our CML lines with PD98085, a specific inhibitor of MAPK signalling, or with LY294002, a specific inhibitor of PI3 kinase, we did not detected any significant decrease in the levels of Survivin. Instead, the treatment with AG490, a specific inhibitor of the JAK2/STAT pathway, caused a decrement in the Survivin expression levels. Finally, when we suppressed Survivin by siRNA, we observed that exposure to hydroxyurea killed most leukemic cells, even in the presence of an active BCR-ABL kinase. Our data suggest that up-regulation of Survivin is a critical step in the reduction of apoptosis that leads to the expansion of the BCR-ABL positive leukemic clone.

PO-117

INDUCIBLE ACTIVATION OF C/EBPβ, A GENE NEGATIVELY REGULATED BY BCR/ABL, INHIBITS PROLIFERATION AND PROMOTES DIFFERENTIATION OF BCR/ABL-EXPRESSING CELLS

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Translational regulation by oncogenic proteins may be a rapid and efficient mechanism to modulate gene expression. We report here the identification of the C/EBPβ gene as a target of translational regulation in myeloid precursor cells transformed by the BCR/ABL oncogene. Expression of C/EBPβ is repressed in 32D-BCR/ABL cells and reinduced by STI571 via a mechanism which appears to depend on expression of the CUG-repeat RNA binding protein CUGBP1 and the integrity of the CUG-rich intercistronic region of c/ebpβ mRNA. Constitutive expression or conditional activation of wild-type C/EBPβ induces differentiation and inhibits proliferation of 32D-BCR/ABL cells *in vitro* and in mice, but a DNA-binding deficient C/EBPβ mutant has no effect. The proliferation-inhibitory effect of C/EBPβ is, in part, mediated by the C/EBPβ-induced GADD45α gene. Since expression of C/EBPβ (and C/EBPα) is low in the aggressive blast crisis (BC) stage of chronic myelogenous leukemia (CML) and is inversely correlated with BCR/ABL tyrosine kinase levels, these findings point to the therapeutic potential of restoring C/EBP activity in CML-BC and, perhaps, other types of acute leukemia.

PO-118

LEUKEMOGENESIS INDUCED BY WILD TYPE AND STI571-RESISTANT BCR/ABL IS POTENTLY SUPPRESSED BY C/EBPA.

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Chronic phase-to-blast crisis transition in chronic myelogenous leukemia is associated with differentiation arrest and downregulation of C/EBPα, a transcription factor essential for granulocytic differentiation. CML-blast crisis patients became rapidly resistant to therapy with the BCR/ABL kinase inhibitor Imatinib (Gleevec; STI571) because of mutations in the kinase domain interfering with drug's binding. We show here that restoration of C/EBPα activity in STI571-sensitive or -resistant 32D-BCR/ABL cells induced granulocytic differentiation, inhibited proliferation *in vitro* and in mice, and suppressed leukemogenesis. Moreover, activation of C/EBPα eradicated leukemia in 4 of 10 and 6 of 7 mice injected with STI571-sensitive or -resistant 32D-BCR/ABL cells, respectively. Differentiation induction and proliferation inhibition were required for optimal suppression of leukemogenesis, as indicated by the more potent effect of p42 C/EBPα than K298E C/EBPα, a mutant defective in DNA binding and transcription activation which fails to induce granulocytic differentiation. Activation of C/EBPα in blast cells from four CML-BC patients including one resistant to STI571 and BMS-354825 and carrying the T315I Abl kinase domain mutation also induced granulocytic differentiation. Thus, these data indicate that C/EBPα has potent anti-leukemia effects even in cells resistant to ATP-binding competitive tyrosine kinase inhibitors and portend to the development of anti-leukemia therapies relying on C/EBPα activation.

PO-119

OVEREXPRESSION OF 14-3-3 SIGMA IS ASSOCIATED WITH TYROSINE KINASE ACTIVITY OF P210 BCR-ABL FUSION PROTEIN OF CHRONIC MYELOID LEUKEMIA.

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The 14-3-3 proteins are a family of phosphoserine/threonine-binding molecules and critical mediators of intracellular signaling pathways, including those controlling proliferation, cell cycle checkpoint activation and survival. In particular, upon c-Jun NH2-terminal kinase (JNK)-mediated phosphorylation in response to stress they release c-abl tyrosine kinase and let its nuclear import, the prerequisite fot its pro-apoptotic and growth arrest function. Here we show that constitutive tyrosine kinase (TK) activity of p210 bcr-abl protein, the molecular hallmark and causative event of Chronic Myeloid leukemia (CML) is associated with overexpression of 14-3-3 sigma. In 32D cell clones transducing a temperature-sensitive bcr-abl construct the levels of 14-3-3 transcript and protein were increased under permissive culture conditions for p210 TK and significantly reduced by p210 TK inhibition by the TK inhibitor Imatinib mesylate (IM). Moreover, in K562 CML cell line we observed the hyperacetylation of a discrete region of 14-3-3 sigma promoter that corresponds to -8245 to -8508, that was significantly reduced since 4th hour of exposure to IM. Conversely, the methylation status at a CpG-rich area of 14-3-3 sigma coding region including the transcription start site (-220 to + 116) was not conditional upon p210 TK. Our results support that p210 TK influences 14-3-3 sigma trascription rate by interacting with epigenetic mechanisms that control chromatin accessibility. Interestingly, in K562 cell line IM resistance was associated with a further increse of 14-3-3 sigma expression and higher hyperacetylation at its promoter, supporting a putative role of this scaffolding protein in clonal evolution of CML progenitors towards drug resistance. Further studies are presently in progress to elucidate mechanisms relevant for 14-3-3 overexpression and enhanced binding properties, whether they may be targeted by drug combinations that have been advanced for clinical trials.

PO-120

GENE EXPRESSION PROFILE OF PATIENTS INNATELY RESISTANT TO IMATINIB MESYLATE

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Background. STI571 (Imatinib, IM) a specific ABL tyrosine kinase inhibitor has been reported to have a significant clinic effect on chronic myeloid leukaemia (CML) in chronic phase as well in blast crisis. However, many patients treated with IM relapse at a relatively early time, suggesting that leukaemia cells tends to acquire resistance to IM. This secondary resistance is probably due to powerful selective pressure on rare cells that carry amplified copies to of the BCR-ABL fusion oncogene or point mutations in the ABL tyrosine kinase domain affecting the binding site of the drug. In others cases resistance appear to exist prior to drug exposure. Such innate mechanism of resistance (primary resistance) is poorly understood but some evidences suggest that activation of alternative oncogenic pathway, may confer BCR-ABL independent survival to CML cells. Comparative genome wide expression studies have long been known to provide important insight into biological process such as proliferation, differentiation, apoptosis and transformation. Only few gene expression profiling-based studies of CML and IM treatment have so far published. Moreover, only three studies has been performed on patient's samples, resulting on heterogeneous conclusions. To investigate about the molecular events involved in innate imatinib resistance in CML we compared the expression profile of a set of 380 genes on samples of resistant patients, respondent patients and healthy donors. We chosen 380 genes known from high density micro arrays expression data to be associated to different CML phenotypes and with relevance in process like apoptosis, cell adhesion, cell proliferation, signal transduction, chromosome/DNA dynamics. *Methods.* A set of 13 patients with CML (median age 50 years, range 21 to 80; 3 pts female and 10 pts. male) was selected from several diagnosed at Division of Hematology of the *Cervello Hospital* of Palermo. As a control for the gene expression experiments we used bone marrow samples from two healthy volunteers. Selection was based on each patient's response to imatinib and the availability of sufficient material. Patients were defined as responder to IM if they achieved a minimal residual disease reduction (level of BCR/ABL trascript) greater than 3 log within 6-12 months, while resistant were defined as those with less than 1 log of reduction of minimal residual disease after 6-12 months of treatment. RNA samples for the array analysis were isolated from total bone marrow white cells. We use as Low-Density Arrays the TaqMan MicroFluidic Card (MFC) system. This technology is a medium-throughput method for real-time RT-PCR that can simultaneously assay the RNA expression levels of up to 380 genes on a single card. The RT-PCR amplification were run on an ABI 7900 HT with a TaqMan Low Density Array System. RT-PCR data were quantified using the SDS 2.1 software and normalized using the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as endogenous control. *Results and Conclusion.* After the analysis of seven responder and six no responder samples we detected different expression of 29 genes that correlate with the imatinib resistant phenotype. The resistant cells over express (1.9-10 fold increase) genes of different categories: signal trasduction (SOS1,PEA15), apoptosis (BCL2, BAX), genes involved in cell adhesion (SELL, IL-17R, ITGB7), genes related to cell cycle progression (CCND2, CDK4) and transcription factors genes (ETS1, ETS2, KLF7, STAT5B). By comparing expression patterns of CML with those of normal cells, we identified 99 genes that were abnormally regulated in CML cells. Among them a lot already reported in previous studies. Their products are involved in regulation of a variety of cell functions including cell cycle (CCND1, CDK4), cell adhesion (PSTPIP1), signalling (LILRB3), DNA replication and repair (POLD2, POLE) and chromosome/chromatin dynamics (MCM3). In this study we identified several genes implicated in cellular process that are disturbed in Ph-positive leukemias, in particular we noted an over-expression in resistant patients cells of STAT5B and CCND2. Notably recent insights into IM mechanism of action revealed that IM repress cyclin D2 by the inhibition of an PI3-K /STAT5 integrated mechanism, blocking, at least, the cell cycle progression. Infact the IM treatment increase BCL6 expression through the inhibition of PI3-K/AKT pathway and BCL6 replace STAT5 at STAT5/BCL6 site in CCND2 promoter repressing CCND2 expression. In this study we identified a large number of genes that were abnormally expressed in CML, confirming previous findings and adding more genes implicated in properties of CML cells. Accumulation of data with respect to expression profiles will help us to better understand the mechanism of carcinogenesis in CML and may also yield practical information about potential target for CML therapeutics.

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PO-121

NEW GENOMIC ISSUES ON DER(9) DELETIONS IN CHRONIC MYELOID LEUKEMIA

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The Philadelphia (Ph) chromosome is found in more than 90% of chronic myeloid leukemia (CML) patients. Deletions adjacent to the translocation junction on the derivative chromosome 9 were described by several groups. These studies revealed two main points:1) genomic microdeletions were concomitant to the t(9;22) translocation; 2) deleted sequences were located upstream to ABL and downstream to BCR genes. We report a detailed molecular cytogenetic characterization of chromosomal rearrangements in two CML cases bearing deletions on der(9) without the characteristics reported above: a complex variant t(9;22) and an ins(9;22)(q34;q11) were detected in cases #1 and #2, respectively. Both patients were diagnosed and tested by conventional cytogenetic analysis, fluorescence in situ hybridization (FISH), and RT-PCR. FISH identification of the ABL and BCR genes was performed using a pool of PAC, RP5-835J22 and RP5-1132H12, and the BAC RP11-164N13, respectively. A set of BAC/PAC probes (proximal and distal to ABL and BCR, respectively) belonging to 9 and 22 chromosomes allowed us to define precisely the deletion size. The UCSC database (University of California Santa Cruz, http://www.genome.ucsc.edu) was queried for BAC/PAC probe locations and for gene identification. FISH experiment performed on case #1 metaphases with BCR and ABL specific probes revealed one fusion signal on der(22) chromosome, a faint ABL signal on der(9) and a split BCR signal on der(6) and on der(12) chromosomes. Reiterative FISH experiments using appropriate BAC/PAC clones, allowed the precise definition of the complex rearrangement breakpoints. Surprisingly, the detailed molecular cytogenetic characterization of chromosome 9 breakpoint showed genomic loss of about 400 Kb downstream to ABL gene. NUP214 is the alone gene with known function mapping in the deleted region. According to our FISH results, the revised karyotype was the following 46,XX,t(6;9;12;22)(p22;q34;q13;q11). Conventional cytogenetic analysis performed on case #2 revealed a normal karyotype. FISH analysis with clones specific for ABL and BCR genes showed a single fusion signal on der(9). These results suggested the occurrence of a cryptic insertion generating a 5'BCR/3'ABL fusion gene on the der(9) instead of 22q11. Further FISH experiments using clones located proximally to BCR showed that a chromosome 22 region of 3 Mb was inserted on 9q34. The use of BAC clones proximal to ABL and distal to BCR showed the loss of chromosomes 9 and 22 sequences on der(9). Two known and one candidate tumor suppressor genes (TSGs) map in the deleted regions: SMARCB1 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin subfamily B member 1) and GSTT1 (glutathione S-transferase theta 1) in 22q11, and PRDM12 (PR domain containing 12) in 9q34. In conclusion, our data indicate that deletions on der(9) in CML cases could also involve chromosome 9 sequences located telomeric to the ABL gene apart from the centromeric sequences previously described. Moreover, genomic microdeletions can be associated to rearrangements involving 9 and 22 chromosomes, such as insertion event, other than reciprocal translocation.

PO-122

A COMPREHENSIVE ANALYSIS OF PRE-EXISTING BCR-ABL GENE ATP-BINDING DOMAIN MUTATIONS ASSOCIATED WITH NATURAL GLEEVEC RESISTANCE IN CHRONIC MYELOID LEUKEMIA (CML) PATIENTS

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Introduction. Imatinib (Novartis) is regarded as the most successful treatment for CML patients at all disease stages. In most of the patients treated with imatinib, resistance has been reported (acquired resistance) making Imatinib resistance as one of the most emerging issues of pharma-

cogenomics.Point mutations in ATP-binding domain of BCR-ABL fusion gene has been detected in resistant patients which disturb the binding of Imatinib to its target bcr-abl oncoprotein, leading to resistance. Studies show that in some cases, mutations pre-exist the therapy and lead to natural Imatinib resistance. Detection of pre-existing mutations can help in deciding the potential responders and poor responders of the treatment and can help in adjusting the treatment accordingly, thus leading to personalized medication. In this study, pre-existing BCR-ABL ATP domain mutations were detected in CML patients who developed Imatinib resistance, later on i.e. on initiation of the therapy. *Material and Methods.* A very sensitive ASO-PCR was used to detect point mutations in CML patients prior to Imatinib therapy. Upon initiation of therapy, follow-up studies were carried out to know the Imatinib resistance. *Results.* A total 104 patients were studied for three mutations. Mixed mutations were detected in 37 (35%), mutation T1052 in 30 (28%), mutation T932C in 18 (17%) and mutation C944T in 14 (12%) patients. All patients showed resistance to Imatinib after therapy initiation. *Conclusions.* This research report shows that mutations in BCR-ABL ATP-binding domain exist prior to therapy and mutant clones probably proliferate after therapy initiation, leading to natural Imatinib resistance.These findings of all great clinical importance in managing resistance to imatinib and other such drugs and open ways for personalized medication. It will further lead towards understanding molecular mechamisms of resistance to molecularly targeted cancer therapies and to develop the strategies not only to overcome this resistance but also to design more effective drugs.

PO-123

CODING SEQUENCE AND INTRON-EXON JUNCTIONS OF THE C-MYB GENE ARE INTACT IN THE CHRONIC PHASE AND BLAST CRISIS STAGES OF CHRONIC MYELOID LEUKEMIA PATIENTS

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The c-myb gene encodes a transcription factor required for proliferation, differentiation and survival of normal and leukemic hematopoietic cells. c-Myb has a longer half-life in BCR/ ABL-expressing than in normal cells, a feature which depends, in part, on PI-3K/Akt-dependent regulation of proteins interacting with the leucine zipper/negative regulatory region of c-Myb. Thus, we asked whether the stability of c-Myb in leukemic cells might be enhanced by mutations interfering with its degradation. We analyzed the c-myb gene in 133 chronic myeloid leukemia (CML) patients in chronic phase and/or blast crisis by Denaturing High-Performance Liquid Chromatography (D-HPLC) and sequence analysis of PCR products corresponding to the entire coding sequence and each exon-intron boundary. No mutations were found. We found four single nucleotide polymorphisms (SNPs) and identified an alternatively spliced transcript lacking exon 5, but SNPs frequency and expression of the alternatively spliced transcript were identical in normal and CML cells. Thus, the enhanced stability of c-Myb in CML blast crisis cells and perhaps in other types of leukemia is not caused by a genetic mechanism.

PO-124

SPHINGOSINE KINASE 1 IS INVOLVED IN SURVIVAL AND PROLIFERATION OF MYELOID LEUKEMIA CELLS, REPRESENTING A POTENTIAL NOVEL TARGET FOR TREATMENT

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Sphingolipids (SPLs), classically known as structural components of cellular membranes, are emerging as bioactive mediators of different cellular processes. New data are available on SPLs as promising therapeutic tools alone or in combination with other compounds, as well as possible valid targets to induce death in leukemia cells and to overcome drug resistance. In this direction, an important role has been attributed to deregulation of sphingosine kinase 1 (SK1) that phosphorylates sphingosine (Sph) to Sph-1P, which in turn promotes cell survival and proliferation. Aims of this study were to verify whether SK1 is indeed involved in the growth and survival of myeloid leukemia cells, therefore representing a potential novel target for treatment. As *in vitro* models, cell lines representing different subtypes of myeloid leukemia were used [AR230 (p230Bcr/Abl+), K562 and RWLeu4 (both p210Bcr/Abl+) for chronic myeloid leukaemia (CML), HL-60 for acute promyelocytic leukaemia and Eol-1 for hypereosynophilic leukemia]. Furthermore, primary peripheral blood mononuclear cells from 5 patients with CML (4 with the p210 and 1 with the p230 isoform of Bcr/Abl), collected at the time of diagnosis and after treatment with Imatinib at the achievement of complete cytogenetic remission (CCR) were analyzed. The basal level of expression and the activity of SK1 were measured by semi-quantitative RT-PCR and a radioactive assay, respectively. The WST-1 assay was used to evaluate *in vitro* cell proliferation with and without the addition of the commercially available drug called SK Inhibitor (Calbiochem), designed to specifically inhibit SK1. Although different levels were identified in the various cell lines, a correlation between the expression and the activity of SK1 was observed. The addition of SK Inhibitor caused an evident dose- and time-dependent decrease of cell proliferation and viability, together with a significant decrease of SK1 activity. To further confirm the association between SK1 inhibition and cell growth arrest, experiments of silencing SK1 gene by specifically designed siRNA are currently ongoing. A variable expression of SK1 was detected in all CML patients at the diagnosis, with a median SK1/GPDH ratio of 0.46. At the time of CCR (achieved after 3 to 4 months of treatment with Imatinib 400 mg/die) in the four patients carrying the p210 Bcr/Abl, an average 5-fold increase of SK1 expression was observed compared to the level measured at diagnosis, whereas no difference was detected in the patient with the p230 isoform. Although preliminary, our results indicate that SK1 does have a role in survival and proliferation of different subtypes of myeloid leukaemia cells. Therefore, pharmacological inhibition of SK1 represents a possible novel target for leukemia treatment. The observation that levels of expression of SK1 in patients with p210 Bcr/Abl-positive CML consistently increase under treatment with Imatinib warrants further investigations.

PO-125

TYROSINE KINASE INHIBITOR STI571 (IMATINIB) COOPERATES WITH WILD-TYPE P53 ON K562 CELL LINE TO ENHANCE ITS PROAPOPTOTIC EFFECTS.

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In order to ascertain whether p53 has a role in chronic myeloid leukemia hematopoietic progenitor response to the innovative tyrosine kinase inhibitor STI571 (Imatinib), we overexpressed a wild type (wt) p53 construct in the K562 cell line, generated from a human blast crisis and lacking endogenous p53. Wt p53 overexpression was associated with a significant reduction of bcr-abl expression levels resulting, at least in part, from post-transcriptional events affecting the stability of p210 bcr-abl fusion protein. Moreover, we demonstrated that p53 overexpression enhances the commitment to the apoptotic death fate of K562 following its *in vitro* exposure to 1 microM STI571. Multiple mechanisms are involved in p53 impact on K562 survival: Most importantly, we found that a greater reduction of bcr-abl transcription by STI571 was associated with the overexpression of wt p53. Further studies are required to elucidate the mechanisms involved in the transcriptional repression of bcr-abl by STI571 and p53 and in their synergic effects on the clonal hematopoiesis of chronic myeloid leukemia.

PO-126

P210 BCR-ABL TYROSINE KINASE INTERACTS WITH HISTONE DEACETYLASE 1 IN CHRONIC MYELOID LEUKAEMIA HAEMATOPOIETIC PROGENITORS: CONSEQUENCES ON HISTONE H4 ACETYLATION AND CHROMATIN STRUCTURE

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The BCR-ABL fusion gene originated from balanced (9;22) translocation is the molecular hallmark and the causative event of Chronic Myeloid Leukaemia (CML). The interactions of its p210 protein constitutively activated and improperly confined to the cytoplasm with multiple regulatory signals of cell cycle progression, apoptosis and self-renewal induce the illegitimate enlargement of clonal hematopoiesis and genetic instability that drives its progression towards the fully transformed phenotype of blast crisis. However, its effects on the basic transcription machinery and chromatin remodeling are unknown. Our study underscored histone H4 hyperacetylation associated with p210 tyrosine kinase (TK) *in vitro* and *in vivo* and its role in BCR-ABL transcription. Histone H4 acetylation status was assayed in 32D murine myeloid progenitor cell line expressing a ts BCR-ABL mutant by labelling immunoprecipitated (IP) chromatin (CHIPs) with an anti-Ac-H4 antibody. Under permissive culture condition for p210 TK (33°C), histone H4 acetylation was reduced between 4 and 24 h of imatinib mesylate (IM) exposure concomitantly with p210 dephosphorylation and enzimatic activity reduction. *In vivo* histone H4 acetylation signals on CHIPs of CD34+ progenitors from CML patients at diagnosis were more intense than those of normal controls and were significantly reduced at day 15 of IM therapy. To address the putative p210 TK role on histone H4 methylation *in vitro* and *in vivo* advanced by mass spectrometry analyses we proved that histone H4 trimethylation at Lys20 was significantly reduced in presence of p210 TK and restored after p210 TK inhibition by IM *in vitro*. Histone H4 hyperacetylation associated with p210 TK *in vitro* and *in vivo* proceed, at least in part, from Hdac1 loss of function arising from its cytoplasmatic compartmentalisation by p210 TK. Indeed p210 TK is associated with histone H4 hyperacetylation at a BCR promoter region (-40 to +285) critical for BCR-ABL transcription in LAMA cell line. BCR-ABL transcript levels were reduced by approximately 20% at 4 h of IM exposure and further declined to 40% of untreated control at 24 h. Amplification signals of DNA from anti-Ac-H4 CHIPs were significantly reduced at 2 h of IM exposure and remained lower compared with untreated control up to 24 h. Complementary activities are probably implicated in the control of histone H4 acetylation status relative to p210 TK.

PO-127

HYPOXIA SUPPRESSES BCR/ABL AND SELECTS IMATINIB-INSENSITIVE PROGENITORS WITHIN CLONAL CML POPULATIONS.

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We previously showed that resistance to severe hypoxia defines hierarchycal levels of progenitors within normal hematopoietic populations and enhances the maintenance of stem cells. This study addressed the questions whether (a) cells endowed with different levels of sensitivity to hypoxia were identifiable within clonal chronic myeloid leukemia (CML) cell populations, and (b) hypoxia-resistant cells were related to those responsible for CML resistance to the treatment with Imatinib-mesylate. CML cell lines were found to comprise hypoxia-sensitive colony-forming cells as well as hypoxia-resistant progenitors of the shortterm repopulating type. The latter were completely Imatinib-insensitive and lacked BCR/Abl protein, but not mRNA, expression. This is the first study directly linking hypoxia-resistance of CML progenitors to BCR/Abl-independence and Imatinib-insensitivity and pointing to a metabolic peculiarity which is relevant to the maintenance of cancer stem cells in solid tumors.

Chronic Myeloid Leukemia II

PO-128

EFFECTIVE TARGETING OF MEK/ERK SIGNALLING BY THE HIGHLY SELECTIVE SMALL MOLECULE INHIBITOR OF MEK, PD0325901, IN HEMATOPOIETIC AND SOLID TUMORS

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In the MAPK module, MEK lies upstream of ERK which is fo=und constitutively activated in a host of human tumors and mediates increased proliferation and resistance to apoptosis. We already demonstrated the cytostatic/cytotoxic activity of MEK inhibitors in myeloid cells. PD0325901 is the latest small-molecule inhibitor of MEK characterized by an increased metabolic stability. We tested its activity (0.1 to 1000 nM) in a broad spectrum of leukemia, melanoma and breast cancer cell lines evaluating changes on cell cycle distribution, apoptosis, protein and gene expression profiles. Among hematopoietic cell lines, PD0325901 induced a marked growth inhibition in myeloid cells with constitutive ERK activation (OCI-AML2, OCI-AML3, and HL-60 with IC50 ranging from 5 to 19 nM). The APL cell line NB4 and the multiple myeloma cell line U266 displayed intermediate sensitivity (IC50=822 and 724 nM, respectively). Relative resistance to PD0325901-mediated growth inhibition (IC50 >1 microM) was observed in myeloid cell lines without constitutive ERK activation (U937, KG-1) and in lymphoid cell lines. Among the solid tumor cell lines, the M14 melanoma, which carries an activating mutation in the B-Raf gene, was markedly sensitive to PD0325901-induced growth inhibition (IC50 = 24 nM), even with forced Bcl-2 expression (IC50 ranging from 64 to 200 nM in Bcl-2-overexpressing clones). Conversely, the breast cancer cell lines tested (SKBr3, BT474, MDA-MB-231 and ZR 75-1) proved relatively resistant (IC50 β1 microM), regardless of the ERK phosphorylation status. In responsive cells PD0325901 rapidly (15 min) and persistently (β 24 h) inhibited ERK phosphorylation in a dose-dependent manner. Cell cycle distribution analysis demonstrated a dramatic dose-dependent reduction of cells in S-phase (e.g. 36.8%±2.8 vs 13%±4.5 after 24 h of exposure to vehicle control DMSO or 10 nM PD0325901, respectively, in the OCI-AML3) and accumulation of cells in the G0/G1-phase. Higher drug concentrations and longer time (48-72 h) of exposure induced apoptosis in sensitive cell lines. Semiquantitative analysis of the phosphorylation levels of 18 proteins belonging to different molecular pathways, performed using a Kinexus Custom Phospho-protein screening assay in OCI-AML3 cells after 1 and 6 h of treatment with 10 nM PD0325901, confirmed a profound and selective inhibition of ERK-1/2 phosphorylation (5- to 8-fold reduction). We also analyzed the changes induced by PD0325901 treatment in the gene expression profile of the sensitive cell line OCI-AML3 exposed to DMSO or 10 nM PD0325901, using an Affymetrix platform and the HG-U133A 2.0 GeneChip®. Using a $p<0.05$ and a fold changes β 2.0 cut-off, 16 and 96 genes were found to be differentially expressed after 6 h and 24 h, respectively. In agreement with its cytostatic effect, PD0325901 induced transcriptional changes mostly in genes related to cell cycle regulation, particularly G1 checkpoint and DNA replication (such as Cyclin D1 and D3, Cyclin E, and cdc25a). Finally, we analyzed the *in vitro* effects of PD0325901 in primary cells from 18 AML patients. PD0325901 induced a dose- and time-dependent reduction in viability, accompanied by a statistically significant reduction in the percentage of cells in S-phase ($p=0.01$). Moreover, a statistically significant ($p=0.019$) increase in the percentage of apoptotic cells was observed in 7/10 samples after 168 h of culture in the presence of 100 nM PD0325901. In conclusion, we found that PD0325901 displays a promising growth-inhibitory activity in cells with constitutive ERK activation as well as in primary myeloid cells. This effect is mediated by cell cycle arrest in G0/G1 and, at higher concentrations, induction of apoptosis We further demonstrated that the gene expression profile of OCI-AML3 is profoundly altered by PD0325901 treatment, particularly reflecting changes in genes involved in the MEK-dependent regulation of cell cycle, as well as new genes potentially useful candidates for further investigation.

PO-129

PERSISTENCE OF PH+/CD34+ RESIDUAL STEM CELLS IN CHRONIC MYELOID LEUKEMIA PATIENTS AFTER LONG LASTING TREATMENT WITH IMATINIB MESYLATE

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After 6 years of clinical experience, imatinib is to be considered the actual best not-transplant treatment for chronic myeloid leukemia (CML). Yet emerging problems are the appearance of secondary resistance to the drug and the persistence of residual disease. In addition, discontinuation of the drug exerts always in rapid loss of response. Concerning residual CML, most of clinical studies documents the persistence of molecular disease in the majority of patients in complete cytogenetic remission (CCR) and although the absolute level of bcr-abl transcript may vary over the treatment, yet a molecular complete response during imatinib is of rare observation. If the *quantity* of minimal residual disease (MRD) has been extensively monitored by RT-Q-PCR, few data on the *quality* of MRD in patients have been produced. Recently Bathia et al demonstrated the persistence of quiescent malignant progenitors in patients in CCR after short term imatinib treatment. In particular the authors showed in 12/15 patients studied after a median time of 10 months of imatinib a median of 11% of residual CML CD34+ progenitors in the bone marrow while only 3/15 had no detectable residual CD34+ cells. Less is known about residual Ph+/CD34+ cells surviving even after a prolonged therapy with this targeting drug. Thus, we evaluated the amount of bone marrow residual CD34+ cells in 21 CML patients in stable CCR after a long lasting treatment with imatinib. At the time of evaluation, the patients were on conventional dose (400mg) imatinib for a median time of 48 months (range 36-58 months) having achieved a CCR status within 3 to 6 months of treatment. However all of them still showed molecular disease as detected by nested RT-PCR. Bone marrow CD34+ cell-enriched populations were selected from mononuclear cells using immunomagnetic column separation and were evaluated with a dual fusion extra signal FISH analysis for the presence of bcr-abl Dual Color Extra Signal Probe (LSI bcr-abl ES, Vysis) that is able to detect bcr-abl fusion in interphase nuclei with a false positive signal rate close to 0. A minimum of 100 CD34+ nuclei per each sample were evaluated. Interestingly, in 8/21 patients no Ph+/CD34+ cells were detected, while in the remaining 13/21 patients a median of 2% (range 1-11%) of bcr-abl positive progenitors were still observed. In these small selected series of patients prolonged treatment with imatinib appears to be correlated with a lower, yet detectable, amount of residual bone marrow Ph+/CD34+ cells. The clinical significance of these results as well as the role of this alternative method to monitor MRD in CML during imatinib treatment need to be evaluated on a larger series of patients.

PO-130

INTERFERON THERAPY PRIOR TO IMATINIB CAN ERADICATE PRE-EXISTING BCR-ABL ATP-BINDING DOMAIN MUTATIONS CONFERRING NATURAL IMATINIB RESISTANCE IN CML PATIENTS

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Introduction. Imatinib is regarded as first choice therapy for CML patients. It directly inhibits bcr-abl oncoprotein by binding to its ATP-binding domain. Majority of the patients treated with imatinib show resistance to Imatinib therapy (acquired imatinib resistance), leading to relapses. In most of the cases, Imatinib resistance has been attributed to point mutations in ATP-binding domain of BCR-ABL oncogene. It has been authoritatively reported that such mutations may exist prior to imatinib therapy and lead to natural Imatinib resistance, which is more more difficult to manage than acquired resistance. As a better treatment strategy, an alternative drug should be given to such patients to eradicate pre-existing mutant clones before Imatinib. In our research report, we found two CML patients in which Interferon therapy eradicated the pre-existing mutations. *Materials and Methods.* An in-house, sensitive ASO-PCR method was employed to detect pre-existing BCR-ABL point mutations in CML patients in chronic phase. After six months of interferon treatment (patients were non-affording to Imatinib treatment), same method was again employed to note any mutations. Interferon treatment was followed by Imatinib therapy funded by an NGO. *Results.* Mutations were found in CML patient prior to Imatinib therapy. However, we were not able to detect same mutations after six months of interferon treatment. Due to elevated TLC count, Interferon therapy was replaced by Imatinib therapy. Patients showed haematologic and cytogenetic response to Imatinib for two years. *Conclusions.* Present study is a very interesting clinical observation of eradication of pre-existing mutant clones by interferon which can lead to imatinib resistance. It has been proved that such mutant clones can not be eradicated by sustained Imatinib therapy but can lead to much complex resistance, leading to advanced disease stage. Natural Imatinib resistance is one of the biggest challenge of pharmacogenomics. This clinical study can open new ways to devise strategies to study and manage resistance to molecular targeted therapies like Imatinib.

PO-131

EVIDENCE FOR THE OCCURRENCE OF THE PH1 CHROMOSOME AS A SECONDARY EVENT IN PATIENTS WITH CML TREATED WITH IMATINIB.

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Background. Treatment with Imatinib mesilate, an inhibitor of the tyrosine kinase encoded by the BCR/ABL fusion gene resulting from the t(9;22) translocation, is able to induce 95% hematologic and 83% cytogenetic responses in previously untreated patients with Chronic Myeloid Leukemia (CML). Chromosome studies have documented the occurrence of clonal chromosome abnormalities in Ph-negative cells during Imatinib treatment in about 15% of responding patients, but occurrence of clonal abnormalities together with the Ph1 at diagnosis and their behaviour during Imatinib therapy have been rarely described. We report here the chromosome data of 5 Ph1+ CML patients who showed chromosome abnormalities, additional to the Ph1, at the onset of the disease.

Table 1.

Pts (sex, age)	*Date m/d/y*	*Karyotype*
M.D. (M, 65)	10/10/00	46,XY,Ph1 [9]/46,XY, Ph[1],t(8;21) [9]
	03/27/01	46XY [20]
	05/05/02	46,XY,Ph[1],t(8;21) [24]
	01/13/03	46,XY,Ph[1],t(8;21),+22q- [20]
	05/05/04	Death in BC
M.M. (F, 77)	04/12/02	46,XX,Ph[1],dup(1q) [20]
	08/01/02	46,XX,dup(1q) [20]
	05/07/03	Idem [20]
	10/10/03	Idem [20]
	05/12/04	Idem [20]
	11/13/04	Idem [20]
	07/21/05	Idem [20]
B.A. (M,77)	07/11/03	46,XY,Ph1,del(5q) [20]
	11/15/03	46,XY [15]/46,XY,Ph1,del(5q) [3]/ 46,XY,del(5q) [2]
	04/02/04	46,XY [9]/46,XY,Ph1,del(5q) [11]
	07/21/04	46,XY [10]/46,XY,Ph1,del(5q) [6]/46XY,del(5q)[4]
	10/14/04	46,XY [15]/46,XY,del(5q)[2]/46,XY,Ph1,del(5q) [3]
	01/15/0	46,XY [15]/46,XY,del(5q) [5]
	07/08/05	46,XY [16]/46,XY,del(5q) [4]
F.D. (M, 65)	10/24/02	46,XY,Ph1 [16]/47,XY, Ph1,+8 [8]/47,XY,+8 [4]/46,XY[2]
	03/05/03	47,XY,+8 [14]/ 46,XY[8]
	06/15/03	47,XY,+8 [22]
	09/10/03	Death in BC
B.P. (M, 80)	01/12/0	46,XY,Ph1,+14 [22]
	404/18/05	47,XY,+14 [16]/46XY [4]
	01/16/06	47,XY,+14 [14]/46XY [6]

During Imatinib treatment, we observed the complete or partial disappearance of the Ph1 cells and the emergence of Ph-negative cells containing the additional abnormality detected at diagnosis. *Methods.* The data are the result of a collaborative study in which all the laboratories involved in the GIMEMA WP on CML study have been asked about the occurrence of such cases. *Results.* Five patients with the features requested have been found. Their serial karyotypes during Imatinib therapy are shown in Table 1. *Discussion.* Our observations support the hypothesis that non random chromosomal aberrations revealed by Imatinib treatment belong to clones with different growth potential, deriving from a common, Ph1-negative, progenitor cell. This would indicate that the occurrence of the Ph1 chromosome may be, at least in some cases, a secondary event. In pt n.4 the same abnormality, trisomy 8, was detected at diagnosis both in Ph1+ and Ph- cells, suggesting that it occurred before any other chromosome change. Consequently, the Ph1 occurred both in a normal cell and in one harbouring a trisomy 8, indicating a secondary and biclonal origin of the Ph1+ cells. Moreover, the blastic crisis of this pt was likely sustained by the Ph1-negative, +8 cells already detected at diagnosis. This observation supports a selective but not causative role for Imatinib in the Ph1-negative clonal evolution.

PO-132

ERADICATION OF IMATINIB-RESISTANCE-CONFERRING, PRE-EXISTING BCR-ABL ATP-BINDING DOMAIN MUTANT CLONES BY SUSTAINED INTERFERON THERAPY IN CML PATIENTS

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Imatinib (Novartis) is currently first-line therapy for CML patients in all stages. It binds to BCR-ABL ATP-binding domain of bcr-abl oncoprotein. Majority of the patients treated with imatinib show resistance to Imatinib therapy (acquired imatinib resistance), leading to relapses.Imatinib resistance has been attributed to point mutations in ATP-binding domain of BCR-ABL. Recent studies showed that in some patients, such mutations exist prior to imatinib therapy and lead to natural Imatinib resistance. An alternative drug should be given to such patients to eradicate pre-existing mutant clones before Imatinib otherwise complex patterns of Imatinib resistance may be exhibited which will be much difficult to managed. We observed two CML caes of in which Interferon therapy eradicated the pre-existing mutations. An in-house, sensitive ASO-PCR method was employed to detect pre-existing BCR-ABL point mutations in CML patients. After six months of interferon treatment, same method was again employed to note any mutation. Interferon treatment was followed by Imatinib therapy. Mutations were found in CML patient prior to Imatinib therapy. However, we were not able to detect same mutations after six months of interferon treatment. Due to elevated TLC count, Interferon therapy was replaced by Imatinib therapy. Patients showed haematologic and cytogenetic response to Imatinib for two years.This is a very interesting clinical observation of eradication of mutant clones by interferon which lead to imatinib resistance. It has been proved that such mutant clones can not be eradicated by sustained Imatinib therapy but can lead to much complex resistance, leading to advanced disease stage. This clinical study can provide a path to manage the resistance to imatinib.

PO-133

FIRST REPORT OF RING CHROMOSOME 21 AND PHILADELPHIA CHROMOSOME (BCR-ABL FUSION GENE) IN A 40 YEARS OLD CML PATIENT RESISTANT TO HYDROXYUREA: RESPONSE TO INTERFERON AND IMATINIB

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Chronic Myeloid Leukaemia (CML) is the most common haematological malignancy. It is usually due to BCR-ABL fusion gene formed as a result of chromosomal translocation t (9;22) which can be detected by RT-PCR and karyotype analysis (philadelphia chromosome). Many other chromosomal changes has been found accompanying BCR-ABL fusion gene. Here we report a ring chromosome 21 with philadelphia chromosome in a 40 years old CML patients who was at hydroxyurea treatment. Ring chromosome and Philadelphia chromosome were detected by karyotype analysis. Presence of philadelphia chromome was also confirmed by RT-PCR based detection of corresponding BCR-ABL fusion transcript. Response to inteferon and imatinib was also noted. This is the first report of ring chromosome 21 accompanying BCR-ABL fusion gene in a CML patient resistant hydroxyurea. Patient showed good response to interferon as manifested by disappearance of ring chromosome 21. Imatinib treatment resulted in complete haematological and cytognetic response. Resistance to imatinib was not found till 2 year follow-up. Our results show favourable prognosis of BCR-ABL positive CML patient having ring chromosome 21, when treated with interferon and imatinib. This is a very interesting finding which may help in understanding biology of CML patients with complex karyotypes and their response to therapy.

PO-134

EFFECTS OF CARBOXYAMIDOTRIAZOLE (CAI) ON IMATINIB-RESISTANT CHRONIC MYELOGENOUS LEUKEMIA CELLS

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Chronic myelogenous leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by the (9;22) reciprocal chromosomal translocation that produces a shortened chromosome 22, the so-called Philadelphia (Ph) chromosome. This rearrangement results in the head-to-tail fusion of the breakpoint cluster region (BCR) gene on chromosome 22 at band q11 with the ABL proto-oncogene located on chromosome 9 at band q34. The fusion gene BCR/ABL encodes for a 210-kDa oncoprotein that exhibits constitutive tyrosine kinase activity leading to its autophosphorylation and to activation of multiple signaling molecules, including p21Ras, signal transducer and activator of transcription 5 (STAT5), and phosphoinositide 3-kinase (PI3-kinase). As a consequence, BCR/ABL leads to increased proliferation and inhibition of apoptosis. Moreover, the expression of BCR/ABL converts growth factor-dependent cell lines to growth factor independence. Multiple lines of evidence have established that the BCR/ABL tyrosine kinase is central to the pathogenesis of CML. Imatinib mesylate (Gleevec, STI571), an orally administered selective inhibitor of the BCR/ABL tyrosine kinase, has significant activity in all stages of the disease, inducing hematologic and cytogenetic responses in a high percentage of patients in chronic phase of the disease. However, patients in more advanced phases of CML (blast crisis) frequently develop resistance to Imatinib monotherapy. Actually, the identification of new therapeutic agents that inhibit signaling pathways activated in CML cells represents an important goal. The aim of the present work was to assay the citotoxic effect of Carboxyamidotriazole (CAI), an inhibitor of calcium-mediated signal transduction, on imatinib-resistant CML cells. CAI is a promising new cytostatic anti-cancer drug which has entered Phase II clinical trials at the National Cancer Institute, NIH, USA and for which multiple modes of action have been proposed. Exposure to CAI concentrations of 1-10 μM (0.4-4.0 μg/mL) has anti-tumor activity, inhibiting tumor cell calcium-mediated signal transduction and proliferation *in vitro*, inhibiting invasion *in vitro* and *in vivo*, and reducing both the number of spontaneous metastases and the tumor burden in mice receiving oral drug administration. The experiments were carried out on three imatinib-resistant cell lines (LAMA84R, K562R and KCL22R) characterized by different mechanisms of resistance. Our results show that CAI induces in all three cell lines a dose-dependent reduction of proliferation rate and a negative modulation of phosphotyrosine protein pattern. Moreover, further analysis of specific signal transduction pathways indicates that CAI is able to inhibit both BCR-ABL-dependent and independent signaling pathways. On the basis of these preliminary results concerning the effects of CAI on imatinib-resistant CML cells, we have performed a comparative study of the proteomic profile of treated and no-treated cells. This approach allowed to detect variations in levels of expression of several proteins, metabolic enzymes and molecular chaperones. We believe the proteomic approach a power tool to understand the molecular mechanisms affected by CAI in CML cells, providing important information for a next rational use of this drug in treatment of patients not responsive to imatinib monotherapy.

PO-135

MONITORING CYTOGENETIC AND MOLECULAR RESPONSE IN CHRONIC MYELOID LEUKEMIA AFTER IMATINIB MESYLATE TREATMENT

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Chronic Myeloid Leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by the acquisition of the Ph chromosome/BCR-ABL fusion oncogene. Imatinib mesylate inhibits the tyrosine-kynase activity of the BCR/ABL fusion gene product and is highly effective in the treatment and management of Ph-positive CML.A complete cytogenetic response (CCR) is achieved in 50% to 60% of patients treated with imatinib after failing IFN-α therapy and in 75% to 90% of those treated with imatinib as their first line of therapy. The cytogenetic response is a predictor of outcome and in quantitative molecular analysis, BCR/ABL transcript continues to decline in CCR. Few cytogenetic relapses have been described after CCR. Major molecular remission (MMolR), as defined by a 3-log reduction in leukemic cells, relates with good prognosis and low risk of disease progression. We studied the clinical, cytogenetic and molecular features of 48 CML patients in chronic phase and accelerated phase after imatinib treatment. Standard cytogenetic, FISH, RT-PCR and RQ-PCR analysis were used to monitor Ph-chromosome/BCR-ABL levels in 24 patients in early chronic phase (CP), 19 in late CP and 5 in accelerated phase (AP). The median follow up on imatinib treatment was 49.86 months (range 12,57-74,67). At standard cytogenetics one patient had a masked Ph and two cases showed a variant three-way translocation. Clonal evolution was observed in six late CP patients (trisomy 8, -Y, 17p-, double Ph), and in 1 accelerated case (-Y). Six patients (4 CP and 2 AP) had a deletion of der(9) at FISH analysis. 36 patients obtained a CCR (1 in AP, 35 in CP); this was subsequently lost in 3 CP patients (one stopped the drug for hepatic toxicity); 3 had a partial cytogenetic remission. At RQ-PCR analysis, we found a MMolR in 22 (only 1 in AP) cases, of which 4 showed the absence of any detectable BCR-ABL transcript. The development of clonal cytogenetic abnormalities in Ph-negative cells has also been observed during imatinib treatment. We identified 2 patients in CCR with a transient clonal cytogenetic abnormality in Ph negative cells were identified: loss of chromosome Y and monosomy 11. The incidence of this phenomenon appears to be quite low but these patients should be followed with conventional cytogenetics even after induction of CCR. In unselected CP-CML patients, imatinib achieved high rates of stable CCR (81.4%) and MMolR (48.8%) with good tolerability. Complete molecular remission (CMolR) as defined by the absence of any detectable BCR/ABL transcript in at least two samples at three months distance, analyzed with both quantitative and qualitative molecular methods (Colombat M, Hematologica 2006), is usually observed only in patients who underwent a allogenic bone marrow transplantation and is uncommon in imatinib treated cases. We observed a stable CMR in 9.3% of the CP cases and this finding was confirmed in all cases over a 3 years period. All four patients with undetectable BCR/ABL transcript received a period of treatment with interferon, either before starting imatinib or as associated treatment. The role of the associated treatment with interferon in these cases remains to be clarified.

PO-136

COMBINED TREATMENT WITH IMATINIB + LOW-DOSE ARA-C IN SIX CML PATIENTS RESISTANT TO IMATINIB ALONE

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Imatinib mesylate represents the front-line treatment for newly diagnosed chronic myeloid leukaemia. Indeed, patients treated at standard dose (400 mg/day) in early chronic phase (CP) have 64-88% chances - depending on Sokal prognostic score - of obtaining a complete cytogenetic response (CCR). Faster cytogenetic responses and a deeper reduction of BCR-ABL transcript have been recently reported, even in high-risk disease, with higher imatinib doses (600-800 mg/day). Nevertheless, a significative proportion of patients are non responders or develop resistance to imatinib, due to BCR-ABL overexpression, additional chromosomal abnormalities or, more frequently, mutations in the ABL kinase domain. *In vitro* studies demonstrated a synergism between imatinib and other antileukemic drugs such as ARA-C and α interferon. Therefore, since 2002 some phase I and II studies with imatinib plus ARA-C have been performed in CML patients unresponsive to IFN or previously untreated; however, reported results did not differ significantly from those obtained with imatinib alone. Here we report the results obtained with an association of imatinib and low-dose ARA-C in six CML patients (five females, one male; five in CP, one in accelerated phase), who had shown partial or complete resistance to imatinib alone administered at a dose of 600-800 mg/day. One patient had displayed primary cytogenetic resistance, two were in hematological relapse, two in cytogenetic relapse (one with a new chromosomal abnormality), whereas one was in CCR but with a persistently high amount of BCR-ABL transcript (BCR-ABL/ ABL ratio: 0.03). Five/six patients had intermediate-high Sokal score at diagnosis (in one patient the Sokal score was not available). Mutations at ABL domain were searched in all six patients and found in one of them, in hematological relapse. Median age was 55 years (range 31-75). All patients continued imatinib therapy at 600-800 mg/day, according to tolerance, with addition of 10 mg/day s.c. ARA-C either continuously (if tolerated) or by 3 week courses. Median duration of treatment was 6,5 months (range 2-16+). Two patients discontinued treatment because of failure, one died of treatment-unrelated cardiac failure after 6,5 months of therapy. The two patients in hematological relapse obtained a complete hematological response: one of them, with documented BCR-ABL mutation (F359V point mutation), also achieved a major cytogenetic response (MCR), the other has not been evaluated yet. Of the two patients in cytogenetic relapse, one reached CCR with major molecular response, the other obtained a MCR. The other two patients didn't obtain any further response by the combined therapy. The treatment was well tolerated, except for grade 2-4 hematological toxicity, requiring temporary reduction/withholding of treatment in three patients. In particular, we observed grade 4 anemia, with marked diserythropoiesis and macrocytosis, requiring blood transfusions in three patients. Anemia then improved with addition of recombinant erythropoietin (40.000 U/week). Three patients experienced grade 2-3 leuko-thrombocytopenia. Grade 2-3 nausea and vomiting was observed in four patients. One patient developed, during disease-unrelated oral anti-coagulant treatment, a reversible subdural haemorrhage, whose relationship with anti-leukemic therapy was unclear. In conclusion, the association of low-dose ARA-C to high dose imatinib seems capable of providing a chance of MCR to some patients relapsed on imatinib alone. Studies on a greater number of patients are obviously needed to confirm these preliminary results.

PO-137

CLINICAL AND MOLECULAR BIOLOGICAL STUDIES ON SUSCEPTIBILITY AND RESISTANCE TO IMATINIB IN PAKISTANI CML PATIENTS: FIRST COMPREHENSIVE REPORT FROM SOUTH-EAST ASIA

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Imatinib mesylate (Novartis) is the first targetted therapy for human cancers. A large no. of reports over the globe have provided evidence that it is curently the most successful therapy for CML patients. This drug stops leukaemogenesis by binding ATP-binding domain of bcr-abl oncoprotein, the molecular entity which causes CML. Despite high success rates, resistance has been reported to Imatinib due to mutations in ATP-binding domain of BCR-ABL oncogene. We here describe resistance and susceptibility to Imatinib in Pakistani CML patients. This is first comprehensive report from South-East Asia. A total of 154 CML patients were studied. BCR-ABL fusion gene was detected by RT-PCR. Resistance and susceptibility to imatinib was noted by haematological studies, molecular analysis of BCR-ABL fusion transcript and by detection of mutations using ASO-PCR. Out of total 152 patients treated with Imatinib, 112 (89%) showed haematological response and 97 (64%) showed molecular response. Patients who did not show molecular response later on developed resistance to Imatinib. BCR-ABL ATP-binding domain mutations were detected in 49 out of 55 (89%) Imatinib resistance patients. This study shows that patients who do not show molecular response to Imatinib can show resistance to this drug even if they show haematological response.

PO-138

DETECTION OF RING CHROMOSOME 21 AND PHILADELPHIA CHROMOSOME (T 9:22) IN A CHRONIC PHASE CML PATIENT AND ITS RESPONSE TO INTERFERON AND IMATINIB TREATMENTS

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Chronic Myelogenous Leukaemia (CML) is associated with, in most of the cases, philadelphia chromosome [t (9;22)] detectable by karotype analysis. Resulting BCR-ABL fusion oncogene can be detected by RT-PCR. In CML patients, many other chromosomal abnormalities have been found accompanying BCR-ABL fusion gene. However ring chrmosome 21 have never been reported in philadelphia positive CML. In our clinical and molecular genetic studies of CML patients, we found a ring chromosome 21 with philadelphia chromosome in a 40 years old CML patient in chronic phase and resistant to hydroxyurea treatment. Karyotype analysis was brought about to detect ring chromosome 21 and Philadelphia chromosome. BCR-ABL fusion gene corresponding to t(9:22) was also confirmed by RT-PCR. Clinical follw-up studies were carried out to know patient's response to inteferon and imatinib. We here first time report ring chromosome 21 accompanying BCR-ABL fusion gene in a CML patient. Patient, who was resistant to hydroxurea, showed haematological response to interferon. Most important finding was disappearance of ring chromosome 21 due to inteferon therapy. However, philadelphia chromosome persisted. Imatinib treatment resulted in complete haematological and cytognetic response. After two years follow-up of the patient,imatinib resistance and progression to accelerated phase was not reported. In other leukaemias, different ring chromosome have been found to be associated with poor prognosis. However, our finding suggest that ring chromosome 21 in philadelphia positive CML patients may have favourable prognosis. These findings are needed to be confirmed in a more CML patients with ring chromosome. However, incidence of this type of karyotype is very uncommon in CML patients. In any case, this unique and interesting finding will help in understanding biology of CML exhibiting complex karyotypes and will also help in prognosis and treatment.

Multiple Myeloma and Plasma Cell Dyscrasia I

PO-139

PREVALENCE OF *RAS* GENE MUTATIONS IN THE CONTEXT OF A MOLECULAR CLASSIFICATION OF MULTIPLE MYELOMA<*BR*>

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Earlier studies have reported that activating mutations involving *RAS* genes, in particular *NRAS* and *KRAS*, occur frequently in multiple myeloma (MM). The reported prevalence of mutated tumors varies from 10 to 40% at presentation, rising to 70% at relapse, suggesting a role of this lesion in tumor progression. Notably, the occurrence of such mutation in MGUS and indolent tumors is very low. Mutations of *KRAS*, but not *NRAS*, have been found to be associated with higher bone marrow burden and shorter survival. In the present study we investigated the prevalence and type of RAS mutations in MM in the context of a proposed molecular stratification, named as *TC classification*, based on the presence of IGH translocation and dysregulation of cyclin D genes in MM (Hideshima et al, Blood, 2004). The presence of *NRAS* and *KRAS* gene mutations was investigated in a panel of 82 MM at diagnosis, 13 patients with extramedullary myeloma or plasma cell leukemia, 9 patients with MGUS and 4 normal controls. The mutation analysis was performed by RT-PCR and direct DNA sequencing on purified $CD138^+$ plasma cell populations (>90%). The expression levels of the three cyclin D genes in MM patients were derived from the gene expression profiling (GEP) data generated using high-density oligonucleotide arrays. GEP data were analyzed using unsupervised (two-dimensional hierarchical clustering) and supervised (SAM, Significant Analysis of Microarrays) approaches. Mutations were found in 16/82 (20%) myeloma patients, in 2/13 (20%) PCL samples and in none of the MGUS patients. In 11 MM patients the mutation involved the *NRAS* gene at codon 13 (3 patients) and 61 (8 patients), and the *KRAS* gene at codon 12 (4 patients) and 61 (1 patient), respectively. PCL patients were both harboring a *NRAS* mutation at codon 61. Mutations were found in patients included in all TC groups: 4 patients in TC1 (23.5%), 5 in TC2 (28%), 3 in TC3 (11.5%) and 2 patients in both TC4 (12.5%) and TC5 (50%) groups. Although the higher frequency of mutations observed in TC1 and TC2 groups, this finding did not reach a significant statistical level. No significant correlation was found with chromosome 13q deletion, trisomy of chromosome 11, or 1q amplification. Unsupervised analysis of gene expression profiles of the 82 patients did not show any particular evidence of clustering of tumors with *RAS* mutations. A supervised analysis approach, comparing the *RAS* mutated MM (16) cases versus wild-type (66) tumors in the complete dataset as well as in the TC1, 2, 3 or 4 groups, did not allow the identification of any differentially expressed transcript. Our study confirms the previous evidences reported by others and us and indicates that *RAS* mutations did not correlate at significant levels with specific genetic lesions or molecular features in MM.

PO-140

INTEGRATIVE GENOMIC ANALYSIS REVEALS DISTINCT TRANSCRIPTIONAL AND GENETIC FEATURES ASSOCIATED WITH CHROMOSOME 13 DELETION IN MULTIPLE MYELOMA

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Chromosome 13 deletion [del(13)] represents one of the most frequent genomic alterations in multiple myeloma (MM), associated with almost 50% of the patients. Several groups have reported an unfavorable

prognostic role for del(13) in MM, although there is an increasing agreement that its prognostic relevance has to be related to the remarkable association with the presence of non-hyperdiploidy and specific types of IGH chromosomal translocations. To better characterize the biology of del(13), we provide a comprehensive analysis of the transcriptional profiles and the molecular features associated with del(13) in MM patients. The transcriptional profiles of highly purified plasma cells from 80 newly diagnosed MM patients have been generated by means of high-density oligonucleotide arrays (Affymetrix GeneChip U133A) and subsequently analyzed using unsupervised and supervised approaches (two-dimensional hierarchical clustering and SAM, respectively). Chromosomal regions with modulation of the gene expression signals have been identified using a non-parametric model-free statistical method (LAP, locally adaptive statistical procedure). The aneuploidy status was evaluated by fluorescence *in situ* hybridization (FISH) by combining the criteria of two recently proposed FISH approaches (Wuilleme S. et al, Leukemia, 2004; Chng W.J. et al, Blood, 2005). Genome wide profiling data for 10 MM samples have been generated on high-density SNP arrays (Affymetrix GeneChip Human Mapping 10k Xba 142 2.0 arrays) and analyzed for copy number alterations. The differential expression of 87 transcripts (specific for 67 genes), all of them downregulated in del(13)+ group, distinguished del(13)+ from del(13)- MM cases; forty-four genes were localized along the whole chromosome 13, 7 on chromosome 11 and 4 on chromosome 19. The majority of the identified genes resulted involved in protein biosynthesis, ubiquitination and transcriptional regulation. Notably, we identified the putative tumor suppressor gene *RFP2*, mapping at 13q14.3 within the minimally deletion region. An integrative genomic approach, based on the regional analysis of gene expression data using the LAP procedure, allowed detecting chromosomal regions whose modulation in global expression levels could differentiate the del(13)+ patients. In particular, we identified the upregulation of the 1q42 region and the downregulation of the 19p region and of almost the entire chromosome 11. To better clarify these findings, we investigated the specific chromosome regions by FISH, showing a strong relationship between del(13)+ and both the presence of 1q21-1q42 amplifications ($p=6\times10^{-4}$) or the absence of chromosome 11 trisomy ($p=5\times10^{-4}$). Finally, the genome wide profiling of 10 MM patients included in our study confirmed the patterns observed by FISH. In the present study, the combined integrative genomic and FISH approaches evidenced that the presence of the chromosome 13 deletion in MM is specifically associated with distinct types of chromosomal aberrations, which may account for the transcriptional differences between del(13)+ and del(13)- patients.

PO-141

PAN-GENOMIC SCREENING IN PLASMA CELL DYSCRASIA: CRIPTIC IMBALANCES DETECTED BY ARRAY-CGH

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Background and aims. Array-based comparative genomic hybridization (array-CGH) is a relatively new technology that is designed to rapidly screen the entire genome for unbalanced genetic aberrations. We used this technique to perform pan-genomic screening in 16 patients with multiple myeloma (MM) and 4 with *de novo* plasma cell leukemia (PCL) with the following aims: i) to identify the most common yet undescribed genetic lesions; ii) to verify which among these lesions are linked to the natural history of PCD; iii) to establish a preliminary link with clinical outcome. *Methods*. we employed bone marrow samples. Plasma cells were purified by Myltenyi columns in case of less than 20% of tumor contamination. Array-CGH was performed as follows: genomic DNAs, from both the tumor and normal reference cells, labelled with different fluorescent dyes were cohybridized to 1 Mb resolution BAC-arrays (Spectral Genomics Inc. USA). Variations in DNA sequence copy number for each BAC clone was assessed by relative fluorescence signal intensities, in a single hybridisation, providing a locus-by-locus measure of DNA copy-number changes. *Results*. Array-CGH is feasible and effective and is 90% concordant with FISH for known imbalances. The median number of lesions/patient observed in our panel was 17 (4-135). Also the amount of the total genome affected by chromosomal imbalances was highly variable (median 3.9% range: 0.14%-27%). The amount of involved genome did not correlate with the actual number of lesions. A good correlation was noticed between the amount of losses and gains in each patient. Notably PCL do not have a more disrupted genome compared to MM patients as one might expects based on the highly malignant behaviour. Interestingly two patients with a prolonged clinical history of MGUS prior to MM diagnosis had massive presence of losses ad gains. Of 2600 BACs 934 were never affected, 864 were targeted only in one patient (pt), 401 in two pts, 296 in 3-5 pts and only 105 were targeted in six pts or more. These 105 recurrent imbalances could be attributed to 9 different abnormalities. Despite the small series we have identified five yet undescribed recurring imbalances (frequency 30% or more). These lesions involve chromosome 19p13.2, (10 patients), 14q12 (7 patients), 16q12.1, 11q24 and 9q23 (6 patients each). *Conclusions.* a) array-CGH allow effective pan-genomic screening of MM patients; b) the pattern of genetic disruption is highly heterogeneous with a majority of non-recurrent or uncommonly recurrent lesions; c) a number of highly recurrent lesions have been identified that will require assessment for prognostic impact; d) the overall amount of perturbed genome does not seem to correlate with more aggressive disease, and might be the reflection of alternative biologic features.

PO-142

RELEVANCE OF NUCLEAR FACTOR-κ B EXPRESSION IN MULTIPLE MYELOMA

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Nuclear factor-kappa B (NF-κB) is multifunctional transcription factor that regulates different signal transduction pathways such as cell survival and proliferation. A number of tumors display activated NF-κB, which contributes to promote cancer cell growth and resistance to chemotherapeutic drugs. NF-κB has been shown to be constitutively active in multiple myeloma (MM) cells, resulting in increased expression of Bcl-xL and IL-6. Here, we analyzed the nuclear localization of NF-κB in MM cells derived from 9 different patients with MM at presentation and in relapse, as well as in two myeloma cell lines (XG1, RPMI 8226). NF-κB localization was evaluated by either immunohistochemistry or immunofluorescence using a monoclonal mouse anti-human p65 (Rel A) antibody that recognizes the p65 subunit. Surprisingly, nuclear localization of NF-κB was (weakly) detected in only one MM sample from a refractory MM patient, while the other samples, including the MM cell lines, exclusively express the cytoplasmic (inactive) form of NF-κB. We next analyzed the sensitivity of MM primary cells to different doses of the proteasome inhibitor Bortezomib (from 1 nanomolar to 10 micromolar), which is known to antagonizes NF-κB activity. We found a consistent dose- and time-dependent antitumor activity against both chemoresistant and chemosensitive myeloma cells in all the samples analyzed, independently of NF-κaB localization. These results indicate that Bortezomib is active in MM cells regardless the NF-κaB localization and suggest the existence of other molecular targets of proteasome inhibitors in MM.

PO-143

INSULIN-LIKE GROWTH FACTOR 1 (IGF-1) IS OVER EXPRESSED IN MULTIPLE MYELOMA PLASMA CELLS (PC) AND REGULATES THE EXPRESSION OF THE IGF-1 RECEPTOR

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Background. IGF-1 plays an important role in regulating cell proliferation, differentiation, apoptosis, and transformation. Recent studies have shown that IGF-1 is an important survival and growth factor in multiple myeloma (MM). Moreover, IGF-1 down-regulates IGF-1R expression at the transcriptional level, by autocrine or paracrine mechanism. *Methods.* The expression of IGF-1 and IGF-1R was evaluated by Real-time PCR in a series of 53 newly diagnosed MM patients primarily treated

with thalidomide and dexamethasone. For each patient, we isolated the CD138+ cell fraction from bone marrow (BM) sample at diagnosis and, in 24/53 patients for whom material was available, also at the end of induction therapy; both CD138+ and CD138- cell fractions were studied. A pool of donors was used as calibrator. The Mann-Whitney and the Spearman Rank Correlation tests were applied for statistical analysis. *Aim.* Correlate the expression of IGF-1 and IGF-1R with presenting karyotypic features of MM patients and evaluate their relationship with response to therapy. *Results.* Both neoplastic PC and CD138- cell fractions expressed a markedly high levels of IGF-1 (median 145.01 and 3.07, range 0.13-1089.92 and 0.02-103.25, respectively), and low levels of IGF-1R (median 0.76 and 1.77, range 0.04-10.78 and 0.21-13.55, respectively). Expression data resulted very scattered; as a consequence, no differences could be highlighted in IGF-1 and IGF-1R expression, with respect neither to the most common presenting clinical features, nor to the presence of t(4;14) and del(13). According to response to induction therapy (39 responsive and 15 non responsive patients) again, no relationship between the expression of IGF-1 and IGF-1R was pointed out. In order to look for indications of an autocrine negative-feedback regulatory mechanism, we looked for correlations between the expression values of IGF-1 and IGF-1R; however we could not detect any significant inverse correlation, in any analyzed fraction. On the contrary, a significant inverse correlation was highlighted between the CD138+ expressed IGF-1 and the CD138- expressed IGF-1R (p= 0.01, r= -0.33), thus suggesting a possible paracrine effect of PC-produced IGF-1 exerted on CD138- cells, which resulted more enhanced when analysing patients subgroups not harbouring t(4;14) or del(13) (p<0.0001, r=-0.59 and p<0.0005, r=-0.59, respectively). Conversely in t(4;14)+ patients, the correlation between IGF-1 and IGF-1R expression become significantly positive (p= 0.03, r=0.64). After induction therapy, a median IGF-1R increase was observed among CD138+ samples (0.67 vs. 1.3, p=0.03); patients not harbouring del(13) and t(4;14) showed the most relevant increase (0.6 vs. 1.58, p=0.03 and 0.53 vs.1.13, p=0.0005, respectively). *Conclusion.* Our preliminary study confirmed the involvement of IGF-1/IGF-1R pathway in MM pathogenesis; we suggest a paracrine effect of PC-produced IGF-1 on CD138- cells, which seemed to act only in responding patients. The ability to efficiently regulate IGF-1R expression may thus have an important prognostic value. Moreover, a different regulation of IGF-1/IGF-1R pathway may exist between different genetic subtypes. The study of post transduction modifications of the IGF-1R will be needed, in order to get more insight into the relationship between the IGF-1 and IGF-1R expressions and IGF-1R activation.

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PO-144

ANALYSIS OF HYPERDIPLOIDY IN MULTIPLE MYELOMA BY FLUORESCENCE *IN SITU* HYBRIDIZATION

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Karyotypic instability is a hallmark of multiple myeloma (MM). According to the chromosome number pattern, two major groups are recognized in MM: the hyperdiploid (H-MM) group, associated with recurrent trisomies involving non-random chromosomes, mainly 3, 5, 7, 9, 11, 15, 19 and 21, and the non hyperdiploid (NH-MM) group, including the hypodiploid, pseudodiploid and near-tetraploid karyotypes. Several studies have suggested that most recurrent IGH translocations and chromosome 13 deletion are frequently associated with NH-MM patients. These two categories, based on conventional cytogenetics, are approximately equally distributed and a better outcome has been associated with H-MM patients; however, their characterization is hampered by the low proliferative rate of myeloma cells. Recently, two FISH approaches have been proposed by the two major investigation groups in MM (Wuilleme S. *et al,* Leukemia, 2004; Chng W.J. et al, Blood, 2005). In this study we tested the detection ability of both criteria on CD138-enriched plasma cells from 75 patients with newly diagnosed MM for whom no conventional cytogenetic data were available. Both approaches were based on the evaluation of three chromosomes: 5, 9, 15 (Wuilleme S. et al, Leukemia, 2004 and 9, 11, 15 (Chng W.J. *et al,* Blood, 2005), respectively; hyperdiploidy was defined as the gain of at least two of the three considered chromosomes. Two steps of co-hybridization with a specific set of FISH probes were performed for each case. In addition, the most recurrent IGH translocations, 13q14 deletions and additional copies of chromosomes 1, 11 and 19 were investigated by FISH analyses in all available samples. Our results showed high concordance between the two strategies used for the ploidy investigation (66/75 cases, 88%): twenty-eight out of the 66 concordant MM patients (42,5%) showed hyperdiploidy, in agreement with the percentage reported by the previous authors. Our data did not reveal the presence of a significant correlation between chromosome 13 deletion and NH-MM, whereas within H-MM it was clearly distinguishable the presence of two sub-groups, one associated with chromosome 11 gain and the other showing chromosome 1q gain and chromosome 13 deletion. In conclusion our study showed that both FISH approaches are equally effective, allowing a novel validated hyperdiploidy definition compared to the current available methods, such as conventional cytogenetics or DNA content measured by flow cytometry. Therefore, the specific and sensitive FISH test for the definition of ploidy status will provide new insights into our understanding of its prognostic value and risk stratification groups of MM patients.

PO-145

MOLECULAR CHARACTERIZATION OF MULTIPLE MYELOMA CELL LINES: A MODEL FOR AN INTEGRATIVE GENOMICS APPROACH

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The availability of human myeloma cell lines (HMCLs) has significantly contributed to elucidate the molecular and biological aspects of multiple myeloma (MM), such as the identification of the most recurrent IGH translocations and the complex network of cytokines affecting plasma cell growth and angiogenesis. Recently, genes targeted by the chromosomal translocations, as well as the activity of novel candidate specific therapeutic agents, have been investigated in HMCLs. However, it is well known that the establishment in culture *per se* and the continuous passages in culture confer to the HMCLs a progressive independence from growth factors as well as the gain of multiple genetic lesions. The present study was aimed at a detailed characterization of a panel of 23 HMCLs using a genomic integrative approach combining Fluorescence *in situ* hybridization (FISH) and both gene expression and genome-wide profiling. The 23 HMCLs have been characterized for the most recurrent IGH translocations by FISH and RT-PCR analyses. The gene expression profiling have been generated using Affymetrix HG-U133A high-density oligonucleotide arrays. Expression data has been analyzed with unsupervised (e.g., two-dimensional hierarchical clustering) and supervised (i.e., SAM, Significant Analysis of Microarrays) methods. Genome wide profiling data for 17 HMCLs has been generated on high-density SNP arrays Affymetrix Human Mapping 10K Xba 2.0 and subsequently analyzed to investigate copy number alterations. In the panel of 23 HMCLs, 8 lines displayed the t(4;14) translocation, 4 the t(11;14), 5 the t(14;16), 2 the t(6;14), 1 the t(14;20) and 13 the t(8;14), with the consequent deregulation of the respective target genes. The unsupervised analysis performed on the gene expression data showed that only t(4;14) HMCLs could be grouped in a clearly distinguishable cluster. A subset of 6 HMCLs, 4 of which without known IGH translocations, showed the overexpression of the members of the GAGE tumor antigens, previously described as associated to unfavorable tumor progression in MM patients. Interestingly, transcriptional analysis revealed that *MAF* overexpression is not strictly related to the presence of the t(14;16), since its expression was found in 5 cell lines negative for the translocation. In the group of HMCLs overexpressing *MAF* or *MAFB*, the specific deregulation of the known *MAF* target genes, including *CCND2* and *ITGmicro7*, was observed. Finally, the genome wide profiling of the investigated HMCLs revealed the presence of complex karyotypes, the most common aberrations being the gain of chromosome arm 1q and the loss of chro-

mosome arms 1p, 13q and 17p.The present study extends the characterization of most of the known HMCLs, making it possible a more accurate selection as appropriate model of MM for *in vitro* experiments, and provides insights into the characterization of novel potential genetic lesions in primary tumors.

PO-146

IDENTIFICATION OF SPECIFIC TRANSCRIPTIONAL PATTERNS ASSOCIATED WITH HYPERDIPLOIDY IN MULTIPLE MYELOMA

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Karyotypic instability, including numerical and complex structural chromosomal alterations, is strongly associated with multiple myeloma (MM). According to the chromosome number pattern, two major groups are recognized: hyperdiploid (H) tumors, associated with recurrent trisomies involving non-random chromosomes, mostly 3, 5, 7, 9, 11, 15, 19 and 21; and non hyperdiploid (NH) tumors associated with hypodiploid, pseudodiploid or near-tetraploid karyotypes. MM patients are approximately equally distributed between the two categories; notably, the presence of the most recurrent IGH translocations and chromosome 13 deletion appear to be prevalently associated with NH-MM patients, whereas recent evidences have suggested that H-MM correlates with a favorable prognosis. In order to provide a molecular characterization of these two major genetic categories of MM patients, we performed a gene expression profiling analysis on 66 newly-diagnosed MM patients, all characterized by FISH analyses for the most recurrent IGH translocations, 13q14 deletions and additional copies of chromosomes 1, 11 and 19. The ploidy status was investigated by combining two recently proposed FISH approaches (Wuilleme S. *et al,* Leukemia, 2004; Chng W.J. *et al,* Blood, 2005). The gene expression profiles of highly purified MM plasma cells have been generated by means of high-density oligonucleotide arrays (Affymetrix GeneChip U133A) and subsequently analyzed using unsupervised and supervised approaches (two-dimensional hierarchical clustering and SAM, respectively). Chromosomal regions with modulation of the gene expression signals have been identified using a non-parametric model-free statistical method (LAP, locally adaptive statistical procedure). The differential expression of 293 transcripts (specific for 229 genes) distinguished the 28 H-MM from the 38 NH-MM cases. The 208 upregulated genes in H-MM mapped mainly on the chromosomes 3, 5, 9, 11, 15 and 19 involved in hyperdiploidy, while a significant percentage (29%) of the 21 remaining genes (upregulated in NH-MM) were localized on 16q. The identified transcripts have been further validated on a publicly available gene expression dataset of an independent cohort of 64 MM patients for whom the hyperdiploid status was provided (Carrasco *et al.,* 2005). Notably, the global classification rate for the 64 cases (indicating the correct prediction of the hyperdiploidy for the independent set) resulted of 81%, confirming the validity of the identified transcriptional fingerprint. A functional analysis revealed a significant fraction of genes involved in protein biosynthesis (38%), transcriptional machinery and oxidative phosphorylation. Finally, an integrative genomic approach using the LAP procedure further supported these findings, allowing the identification in H-MM patients of globally upregulated regions on the chromosomes 3, 5, 9, 15 and 19 (involved in hyperdiploidy), along with the downregulation of a region on 16q arm. Our data reinforce the importance of combining cytogenetics and gene expression approaches for a better definition of the genetic alterations in MM and provide a molecular and genomic framework for dissection of disease pathogenesis and clinical management.

PO-147

EVOLUTION OF MGUS INTO MYELOMA AND MODIFICATION OF CYTOKINES' AND ADHESION MOLECULES' PLASMATIC LEVELS

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The aim of this study was to evaluate the influence of some cytokines on the evolution of monoclonal gammopathies. Fifty-one patients with multiple myeloma (MM) divided into 3 groups according to disease stage (MM1, MM2, MM3), 60 with monoclonal gammopathy of undetermined significance (MGUS) and 50 healthy controls (C) were studied. The levels of sCD138 (Syndecan-1), TGF-β1, sVCAM-1, IL-13, Fas/APO-1, IL-6, β2-microglobulin (β2-M) and C-reactive protein (CRP) were assayed. Seven of the 60 cases of MGUS (11.6%) evolved into MM3 during the 5-year follow-up; these cases were studied in order to identify any changes in the cytokine network. β2-M and CRP concentrations increased significantly through C, MGUS and the three stages of MM. TGF-β1, sVCAM-1, Fas/APO-1 and IL-6 levels were significantly higher, while IL-13 concentration was significantly lower, in MGUS and in MM than in C ($p<0.001$). The level of sCD138 was significantly lower in MGUS than in C and significantly higher in MM than in C ($p<0.001$). On the other hand, TGF-β1 concentration was significantly higher, while IL-6 and sCD138 concentrations were significantly lower, in MGUS than in MM ($p<0.001$). Only sCD138 was significantly higher in MM3 than in MM1 and in MM2 ($p<0.001$), while the concentrations of all the other cytokines did not differ significantly between MM1, MM2, and MM3. There were no significant differences in cytokine values between MGUS which evolved into MM3 and MGUS which did not evolve. Moreover, in the 7 cases of MGUS which did evolve into MM3, the cytokine levels were not significantly different between the start of the study and at the end of the follow-up. We conclude that syndecan-1, like the better recognised β2-M, CRP and IL-6 markers, has a prognostic value for the evolution of monoclonal gammopathies.

PO-148

SCREENING OF JAK2 V617F MUTATION IN PATIENTS WITH MULTIPLE MYELOMA

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Background. JAKs tyrosine kinases are important mediator of cellular signals between cytokines, receptors and effector proteins. They have 7 structural domains *JAK homology regions* (JH1-JH7) in particular JH1 and JH2: JH1 has kinase activity, while JH2 has a negative-regulatory function on JH1. Recently a somatic mutation in exon 12 of JAK2 has been described in myeloproliferative diseases Philadelphia Chromosome negative as PV, ET and IMF and more recently this mutation has been investigated also in AML, MDS, aCML (BCR-ABL negative), ALL and CLL. JAK2 mutation was identified in a subset of CMML/ aCML, and MDS but not in B-ALL, T- ALL or CLL. This mutation results in a substitution of valine for phenylalanine at position 617 (V617F) in the JH2 domain and leads to constitutive tyrosine phosphorilation and cytokine hypersentivity. Alterations of JAK/STAT signalling molecules with a constitutive activation of STATs have been reported for several lymphoma as in PMBL and cHL and screening for the presence of the mutation has revealed the absence of JAK2 V617F from all cell lines, PMBLs and HLs. *Aims.* We investigated the presence of JAK2 V617F mutation in Multiple Myeloma, a B-cell neoplastic disease characterized by bone marrow infiltration from malignant plasma cells which secrete monoclonal immunoglobulin fragments. Although several parameters such as β2-microglobulin, serum creatinine, hemoglobin, calcium levels or cytogenetics abnormalities have been taken account as predictive factors of the outcome of patients affected by MM, the molecular features of this disease remain still unclear. Cytokines of interleukin 6 family (IL6) which activate the signals transducers gp130, are major survival and growth factors for MM cells. The signal transduction of gp130 involves JAK1, JAK2 and TyK2 and then the downstream effectors comprising the signal

transducer and activator of transcription 3 (STAT3) and mitogen-activated proteine kinase (MAPK) pathways. Some authors found that an inhibitor of JAK2, AG490, suppressed cell proliferation and induced apoptosis in IL-6-dependent MM cell lines. JAK2 kinase activity, ERK2 and STAT3 phosphorylation were inhibited. *Methods.* To detect the JAK2 V617F mutation we performed allele-specific PCR using genomic DNA from peripheral blood samples of 93 consecutive patients affected by MM. All samples were collected after informed consent from 2002 and were mostly taken at diagnosis. *Results.* Patients'charactheristics were: median age at diagnosis 66 years (range 35-88), M/F 51/42, Immunoglobulin (Ig)G 52/93 (56%), IgA 28/93 (30.1%), micromolecular 9/93 (9.7%), IgD 2/93 (2.1%), IgM 1/93 (1.1%), Stage I 10/93 (10.8%), stage IIA 35/93 (38%), stage IIB 2/93 (2.1%), stage IIIA 44/93 (47%), stage IIIB 2/93 (2.1%). All 93 MM samples analyzed were wild type for the JAK2 V617F mutation and presented the only internal control on electrophoresis agarose gel (364 bp) and not the 203 bp product, indicative of JAK2 mutation V617F. *Conclusions.* Thus the mutation of JAK2 V617F is absent in MM and we can suggest that JAK2 mutation V617F does not play a role in the pathogenesis of MM. Given the importance of Jak2 activation in MM, a comprehensive mutational screening of its coding exons is warranted.

PO-149

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)IN THE SERUM OF PATIENTS WITH MULTIPLE MYELOMA AT DIFFERENT STAGES OF DISEASE

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Vascular endothelial growth factor (VEGF) is considered a potent stimulator of angiogenesis. It has been reported that in multiple myeloma (MM) bone marrow angiogenesis parallels tumor progression and correlates with poor prognosis. The aim of the study was to investigate correlations serum levels of VEGF in multiple myeloma (MM) patients with severity of disease. We have studied 19 patients with MM (12F and 7M, mean age 64 years). 11 patients were at stage II and 8 at stage III. Stage of MM was determined according to Salmon-Durie staging criteria. The serum VEGF was measured by means of commercially available enzyme linked (ELISA) assay kits Quantikine, RD Systems Inc.(USA). The mean VEGF serum concentration in healthy persons was 270 pg/mL. The minimal detectable level of this method was 5 pg/mL. The serum level of VEGF in 10 out of 11 patients with stage II did not exceed the level of VEGF in healthy controls with range from 5.38 pg/mL to 183.80 pg/mL. 3 patients with lower level of VEGF have not shown any progression of the disease and have stayed alive for up to 8 years. However, the increased concentration of VEGF was revealed in 1 female. The 12-month-long follow up showed the progression of the disease to stage III. The serum level of VEGF in patients with stage III was increased (range from 285.02 pg/mL to 765.07 pg/mL). In this group all the patients have been resistant to chemotherapy regimes and 3 of the patients have been treated with Thalidomide. *Summary*. The lower concetration of serum VEGF indicates the longer median time to progression. The level of VEGF correlates with severity of disease and is higher at more advanced stage. It is suggestible to consider serum level of VEGF as a potential prognostic factor of MM.

PO-150

β-FGF, IL-6, TNF-α AND VEGF SERUM LEVELS IN NEWLY DIAGNOSIS MULTIPLE MYELOMA PATIENTS TREATED WITH THALIDOMIDE, DEXAMETHASONE AND ZOLEDRONATE

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The observation that increased bone marrow angiogenesis correlates with advanced phases of multiple myeloma (MM), along with the well documented *in vitro* anti-angiogenic activity of thalidomide has provided the rationale of the investigational use of this agent in patients with advanced refractory MM and, more recently, with newly diagnosed disease. The aims of the present study were to investigate the relationship between serum concentrations of angiogenic cytokines and both baseline characteristics and response to therapy in a series of 96 patients with symptomatic MM who were enrolled in the *Bologna 2002* clinical study. All patients were previously untreated and received four months of therapy with thalidomide (200 mg/d), dexamethasone (40 md/ for 4 days, repeated monthly) and zoledronic acid (4 mg/month) in preparation for subsequent autologous transplantation. Serum levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (βFGF), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) were measured at baseline and therapy. For this purpose, laboratory assays were performed using quantitative sandwich ELISA (manufacturer Pierce Endogen and R & D Systems). At baseline, serum levels of bFGF and IL-6 were significantly higher in MM patients than in healthy controls ($p< 0.0001$ and $p= 0.04$, respectively). We found that serum levels of IL-6 were closely related with both TNF-α and VEGF levels ($p<0,0001$ and $p=0,0142$); serum levels of VEGF were significantly correlated with platelet count ($p=0,0015$). On an intent-to-treat basis and using stringent criteria (EBMT criteria), the overall response rate (> or = partial response) to thalidomide-dexamethasone was 85%; 31 patients (32%) showed at least a very good partial remission (VGPR). Among responders, there was no significant change in pre-treatment and post-treatment plasma levels of angiogenic cytokines. In the group of patients who attained at least a VGPR, post-treatment bFGF levels showed a trend towards a reduction in comparison with baseline, but the difference was not statistically significant ($p=0,08$). Additionally, after therapy we observed a significant increase in the levels of VEGF (pre-treatment mean levels 247,66 pg/mL versus post-treatment mean levels 403,10 pg/mL, $p<0,0001$), without any difference between responders and non-responders. The limited number of patients who failed to respond to thalidomide prevented any formal comparison between responders and non-responders with respect to baseline plasma levels After high dose melphalan, we are measuring serum levels of cytokines on the same sample of patients. The results will be reasonably available within two months. Further research in understanding the role of angiogenesis and angiogenic cytokines in newly diagnosed MM, and more properly clarifying their relationship with primary thalidomide therapy is needed.

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PO-151

SERUM FREE LIGHT CHAINS (SFLC) ASSAY: A SUGGESTIVE NEW CRITERIA FOR EVALUATING DISEASE RESPONSE, PROGRESSION AND RELAPSE IN PLASMA-CELL DISORDERS (PD) AND A PROGNOSTIC FACTOR IN MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE (MGUS)

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Introduction. The response rates, according to the criteria defined by Blade et al. (1998), require specific reduction in M protein levels by electrophoresis and immunofixation (IFE); nevertheless such current response criteria have not proved entirely satisfactory for the analysis of disease outcome after therapy. In addition, there are also problems with the current definition of progression in the Blade criteria (a 25% increase in M protein). In this context, we evaluated the new, highly sensitive sFLC assay, as a more stringent criteria for defining response to the various therapeutic options, progression and relapse. We also investigated a predictive role of sFLC in MGUS evolution. Patients (Pts) and methods: Serum protein electrophoresis, IFE, sFLC and plasma cell counts (PCC) were performed at presentation, post-therapy and progression or relapse, in 20 pts: 5 pts had multiple myeloma (MM) secreting intact immunoglobulin paraproteins (IIMM), 2 were iposecretory-IIMM, 4 pts had non secretory MM (NSMM), 5 pts had light chain MM (LCMM) with Bence Jones proteins (BJp) detectable in the urine by standard methods, 1 LCMM had an impaired renal function with anuria, the remaining 3 pts had AL amyloidosis (AL). Patients were treated with different treatment schema, including dexamethasone-thalidomide (DT), DT-PACE Hybrid, melphalan-prednisone-revlimid (MPR) and autologous stem cell transplantation (ASCT). We also compared results in 5 pts affected by MGUS who progressed to MM and 7 pts with stable MGUS. These 2 groups of pts with MGUS were tested for presence of sFLC in baseline and follow-up serum samples (range 1.5-9 years, median 4.6 years). *Results.* IIMM: sFLC concentrations and ratio at diagnosis were within reference values in 1 pt and abnormal in the other 6. In all responsive pts, sFLC fell more rapidly than intact immunoglobulin (Ig). The two

iposecretory pts showed a progressively decreased of sFLC and lower PCC at bone marrow examination, indicating response to treatment, whereas total Ig were almost unvaried. LCMM: The presence of monoclonal paraproteins at diagnosis was detectable by means of sFLC in all 6 pts. After treatment, a remarkable correlation between sFLC and EBMT response criteria, was observed (2 pts were PR, 1nCR, 2 CR, 1MR). Relapse was always indicated by a rapid increase of sFLC. NSMM: At clinical presentation, serum and urine IFE were negative in all pts, while sFLC were abnormal. During follow up, a correlation between sFLC, PCC and clinical events was observed. AL: At presentation, all 3 pts (2 renal AL and 1 cardiac AL), showed abnormal sFLC concentration and ratio with small amounts of BJp. After therapy, chemotherapy in 1 and ASCT in the other 2, a 50% reduction of baseline sFLC correlated with an improvment of organ function. MGUS: Five/7 pts with stable MGUS showed normal sFLC and ratio at diagnosis and at follow-up, 2/7 pts had slightly abnormal sFLC concentrations and ratio at presentation but these changed to normal ratio during follow-up. Four/5 pts with progressing MGUS showed abnormal sFLC and ratio in baseline serum samples; and in 5/5 there was a rapid increase of sFLC concentration and ratio, correlating with progression to MM. Discussion. The shorter serum half-life of FLC (2-6 h) compared to Ig (20 days for IgG) and the greater sensitivity of sFLC compared to BJp, allowed an early indication of response to therapy, progression and relapse in IIMM and LCMM. sFLC concentation and ratio proved to be an useful tool for diagnosis and management of iposecretory MM, NSMM and AL, and showed a significant correlation with PCC. Abnormal sFLC concentrations and ratios were associated with a higher risk of progression in MGUS.

PO-152

ROLE OF SERUM FREE LIGHT CHAIN (SFLC) LEVEL AND RATIO AS EARLY PREDICTORS OF RESPONSE IN MULTIPLE MYELOMA

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Measurement of serum free light chains (sFLC) and the kappa/lambda ratio (FLCr) with the FREELITE assay showed their usefulness in the diagnosis and monitoring of Multiple Myeloma (MM). Furthermore, preliminary data suggest that this assay could be used in order to predict response to chemotherapy. Our study aimed to evaluate if sFLC and FLCr, after 1-2 chemotherapy cycles, could be predictive for final response in 16 patients treated with thalidomide, desamethasone and pegylated lyposomal doxorubicine (ThaDD). Patients median age was 72 (range 57-80 years), median β2-microglobulin level was 4.0 mg/dl, 12 patients (75%)presented stage III disease and ISS resulted β2 in 11 patients (69%). Post-ThaDD (6 cycles) 4 patients (25%) showed a CR, 10 patients (62,5) PR, and other 2 (12,5) an MR, accordingly with EBMT criteria. After 1-2 cycles, 4 patients (25%) achieved normalization of the absolute value of the pathological free light chain and 9 others (56%) presented an absolute value < 100 mg/L, considered as the cut-off for measurable disease with sFLC. Moreover, 3 patients (19%) showed a normalization of the pathological free light chain/non pathological free light chain ratio and 7 others (44%) presented a ratio < 5. As per the final 4 CR: 3 showed a normalization of the values for the pathological free light chain, in all 4 the pathological free light chain was < 100 mg/L, in 2 there was a normalization of ratio levels and, again, all 4 presented ratio levels< 5. Categorizing final responses to treatment as CR vs no CR, the achievement of CR resulted significantly associated with both normalization of the pathological free light chain levels and with values < 100 mg/l (p=0.027 and p=0.018, respectively) and to the normalization or reduction of values at less than 5 of the ratio pathological free light chain/non pathological free light chain (p=0.072 and p=0.019, respectively). Our preliminary data demonstrate that the values of sFLC and FLCr after 1-2 therapy cycles are significantly predictive for final response to treatment. If these data would be confirmed in larger cohorts, this assay could be used to modulate precociously therapy strategies for MM.

Multiple Myeloma and Plasma Cell Dyscrasia II

PO-153

SERUM FREE LIGHT CHAINS: A POTENTIAL MARKER FOR DIAGNOSIS, EARLY ASSESSMENT OF RESPONSE TO TREATMENT AND RELAPSE IN PLASMA-CELL DISORDERS

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Introduction. Monoclonal free light chains(sFLC) are omogeneous kappa or lambda immunoglobulin light chain molecules produced by malignant B cell, or altered plasma cells. Recently a sensitive, latex-enhanced immuno- nephelometric assay for the detection of serum free light chains (sFLC) has been developed and we investigated a possible role of sFLC as useful marker for: - diagnosis and rapid monitoring; - early response to treatment and therapy regimen selection; - early progression of lymphoproliferative disease. *Patients and method.* sFLC (reference values : free kappa 0.33-1.94 mg/dL, free lambda 0.57-2.63 mg/dL, ratio 0.26-1.65 mg/dL) and total serum immunoglobulin were measured by nephelometry, using a non competive particle enhanced immunoassay, and free light chains ratio calculated. Urinary Bence Jones protein (BJP) were determined by immunofixation (IFE). In the last six months all plasma-cell disorders were tested for sFLC, immunofixation, SPE, BJP and plasma cell count (bone marrow aspiration), at baseline and following treatment, when in progression or recurring. We included in this study the following. Intact immunogloblulin myeloma (IIM) : 13 patients, therapy regimen was administered as follows: 6 had ThADD, 2 ThalDEX (they were included in the Bologna protocol courtesy of M.Cavo), 3 in observation and not treated, 1 Thal only, 1 alkeran/prednisone: 3 Micromolecolar Myeloma; 7 Recidivating Myeloma; 1 Non Secretory Multiple Myeloma (NSMM); 1 AL amyloidosis; 6 MGUS Patients at risk for myeloma progression. *Results*. For the 13 IIMM, sFLC and ratio values were abnormal in 11 pts, while in the remaining 2 both were in the normal ranges even if there was an histological diagnosis of MM. The sFLC median value was 175 mg/dL. Following treatment elevated values of sFLC and ratio anticipated earlier a progression and recurrence. Partial or no response to treatment was associated with sFLC chains values that did not decrease significantly as in the case of patient 4 : baseline sFLC 1870 mg/dL, after two VAD sFLC values was 620 mg/dL, that rincreased to 690 mg/dL after the third VAD cycle, another decrease was oserved at after 1-month therapy with ThaDD (690 mg/dL) that further decreased during the second month cycles (670 mg/dL and plasma cell count around 45%). Normalization of sFLC with a value of 70 mg/dL showed only when the patient was treated with EDAP (plasma cell count =15%). Micro molecular Myeloma: in the 3 cases, diagnosed at baseline, sFLC correlated with response to therapy and plasma cells count reduction, even if light chains were observed in urine.In the patients suspected for progression, sFLC increase anticipated recurrence before any clinical evidence was apparent. NSMM: elevated values of sFLC were present, while no abnormalities at SPE and urinary IFE were reported, providing early diagnosis; sFLC values normalized after treatment.(from 370 to 6 mg/dL of free kappa). AL Amyloidosis: the sFLC increase correlated with progression to MM (as per bone marrow aspiration also), with regression, remission of MM and clinical improvement. And, at 1 year from detection and treatment, there was a reduction in the proteinuria in a situation of amyloidosis-induced renal impairment. Recidivating MM: 5 presented an increase in sFLc values (i.e. sFLC values: patient 1,baseline 30 mg/dL -recurrence 340 mg/dL. Patient 3, baseline 160- recurrence 315) correlating with progression, largely in advance on the onset of any clinical symptoms. In 2 other patients only the ratio was abnormal and, in all 7, bone marrow recurrence has been documented (plasma cell count > 30%). MGUS: at 1-year follow up, of the 6 affected patients presenting out of range sFLC values, 2 presented a low risk for progression and other 4 an high risk instead, accordingly to the risk stratification parameters of Rajikumar (Abnormal serum free light chain ratio,M-spike >15g/L,non-IgG M-spike. Risk stratification: low risk group,none of these factors; intermediate group, 1 or 2 of these factors and high risk group all 3). None of the high risk ones have progressed, but observation has to be continued up at least 5 years. *Conclusions.* the shorter half-life of sFLC (2-6 hours) compared to the longer ones of both IgG and IgA (20 and 6 days respectively) and the FLC higher could consent an earlier and reliable identification of response to treatment, recurrence or progression The 2 MM

with normal sFLC an ratio but with an histologically confirmed diagnosis of myeloma fall within the 3-5% of myelomas that do not secrete sFLC. Thus this initial preliminary data suggest tha sFLC, at baseline and during follow up, could be an useful tool for the monitoring of plasma cell disease in the daily clinical practice. If in a larger cohort of patients, these observations should be confirmed, it would eventually be possible to investigate if in selected cases the sFLC assay could reduce the number of bone marrow cytology.

PO-154

A PHASE II TRIAL OF BORTEZOMIB PLUS MELPHALAN, PREDNISONE AND THALIDOMIDE (VMPT) FOR ADVANCED MULTIPLE MYELOMA

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Bortezomib (Velcade™) and Thalidomide are effective for the treatment of refractory multiple myeloma (MM). In newly diagnosed patients (pts), the addition of Thalidomide to the standard oral Melphalan/Prednisone combination significantly increased response rate and event free survival. *Aim.* This study will evaluate the efficacy and the safety of the combination therapy of Velcade™, Melphalan, Prednisone, Thalidomide (VMPT) in advanced myeloma. *Methods.* The V-MPT regimen included 6 five-week courses of oral Melphalan (6 mg/m^2 on days 1-5), oral Prednisone (60 mg/m^2 on days 1-5) and Thalidomide (100 mg/day continuously). Velcade™ was administered by IV bolus on days 1, 4, 15, 22 at three dose levels: in the first cohort (10 pts) at 1.0 mg/m^2; in the second cohort (10 pts) at 1.3 mg/m^2 and in the third cohort (10 pts) at 1.6 mg/m^2. Dose Limiting Toxicity (DLT) was defined as the occurrence of any grade 3-4 non hematological toxicities, a grade 4 neutropenia > a week, or any grade 4 hematological toxicity except neutropenia. *Results.* Thirty pts with relapsed or refractory myeloma have been enrolled, median age 66 years (range 38-79), 67% IgG, 17% IgA, 17% Bence Jones, median micro2 microglobulin 3.4 mg/L (range 0.4-11.8). Fourteen pts were treated with V-MPT as second line therapy, 16 as third line. Twenty pts received prior autologous transplant, 10 conventional chemotherapy and 9 thalidomide-based regimens. After a median of 5 courses, 20 pts (66.7%) achieved an objective response (complete response 16.7% and partial response 50%). Two pts (6.7%) achieved a minimal response and 3 (10%) stable disease. Five pts (16.7%) were refractory to treatment and experienced progressive disease. In the first cohort, 3 DLT were observed (grade 3 pneumonia, grade 3 febrile neutropenia and grade 3 vasculitis); in the second cohort, 5 DLT were observed (grade 3 Herpes Zoster infections, grade 4 thrombocytopenia and grade 4 anemia); in the third cohort 5 DLT were observed (grade 4 thrombocytopenia, grade 3 fatigue, sensory neuropathy grade 3, grade 3 Candida esophagitis). The most common grade 1-2 toxicities were: infections, fatigue, peripheral neuropathy and constipation. After introduction of prophylaxis with acyclovir, no new HZV reactivation was observed. Among the 8 pts with baseline peripheral grade 1 neuropathy before VMPT treatment, 5 worsened (one grade 3). Treatment-related neuropathy developed de novo in 4 pts (one grade 3). *Conclusions.* Initial results showed that VMPT combination is a promising regimen for advanced myeloma.

PO-155

FREQUENCY AND FUNCTION OF REGULATORY T CELLS (TREGS) ARE HIGHLY PRESERVED IN MULTIPLE MYELOMA

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Naturally occurring CD4+CD25+ T regulatory cells (Tregs) are a subpopulation of CD4+ T cells vital to homeostasis and maintenance of tolerance. Tregs play a key role in hampering anti-tumor immune surveillance by regulating T-cell immune responses against tumor cells. Depletion of Tregs brings to improved anti-tumor immune responses in both preclinical and clinical settings. Contradictory data have been reported in the frequency and function of Tregs cells in multiple myeloma (MM). The aim of this study was to characterize MM Tregs from the phenotypic, molecular and functional standpoints. We studied both peripheral blood (PB) and bone marrow (BM) samples from patients with symptomatic MM and from healthy donors. CD4+CD25high Tregs were normally represented in both PB and BM of MM patients and expressed a memory and activated phenotype. No differences were observed between PB and BM Tregs of healthy donors and MM patients, based on the expression of CD45R0, HLADR, CD40L and CD69 cell surface antigens. Flow cytometry was also used to assess the intracellular expression of Foxp3, a transcription factor which has been shown to be crucial for the inhibitory function of Tregs cells. More than 90% of CD4+CD25high T cells were Foxp3-positive in PB and BM of MM patients and healthy donors. CD4+CD25+ T cells were purified from PB of newly diagnosed MM by MACS sorting. These cells were anergic to the stimulation via TCR and they were as effective as normal donor-derived CD4+CD25+ cells in inhibiting the TCR-mediated proliferation of autologous CD4+CD25- counterparts. To investigate whether clonal restriction had occurred in MM Tregs, we studied TCR diversity of CD4+CD25+ and CD4+CD25- cells by determining the reciprocal usage of BV gene segments (TCRBV repertoire) with a novel multiplex polymerase chain reaction assay, Our results demonstrate an highly preserved polyclonal TCRBV repertoire, providing the first evidence in cancer patients that TCR diversity of Tregs is not skewed by the long lasting exposure to tumor cells. Based on these data, we propose that inhibitory signals delivered by the highly preserved Tregs subset in MM can easily overwhelm the effector mechanisms of antitumor immunosurveillance which are deeply compromised in MM. Thus, depletion or neutralization of Tregs should be considered in future trials aimed at controlling the disease in MM patients by immune intervention.

PO-156

HISTOLOGIC EVALUATION IN MULTIPLE MYELOMA PATIENTS DIAGNOSED AS HAVING OSTEONECROSIS OF THE JAWS

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Osteonecrosis of the jaws (ONJ) is a rare complication that has been described in patients who had previously undergone radiotherapy for head and neck cancer or similar disorders. Recently, a growing epidemic of ONJ has been reported and this has been attributed to the widespread use of bisphosphonates for treatment and prevention of bone involvement in multiple myeloma (MM) or solid tumors. In the present study we have retrospectively reviewed all MM patients treated at our Institution who have subsequently presented a clinical picture – namely pain, mucosal swelling, exposed bone and purulent discharge- suggestive for ONJ. From May 2003 to present, 13 patients (6M, 7F) were analyzed, their median age was 66, all patients but two had received autologous stem cell transplantation, either single (n=2) or double (n=8); two patients had relapsed after first-line treatment and were receiving thalidomide-dexamethasone as salvage therapy. All the patients had been treated with bisphosphonates, either pamidronate alone (n=1) or pamidronate followed by zoledronic acid (n=2) or zoledronic acid alone (n=10); median number of bisphosphonate infusions was 22 (range 10-45). Diagnostic work-up included only panoramic and tomographic imaging in 2 patients, while in 11 patients histological examination of surgically removed bone was performed. Surprisingly, in 4 patients (36%) a consistent infiltrate of monoclonal plasmacells was detected, thus suggesting involvement by underlying disease. Complete repair or significant healing of the lesions occurred in 9 patients after antimicrobial therapy, either alone (n=2) or combined with surgery (n=5) or with thalidomide-dexamethasone salvage therapy (2 patients with MM at histological examination). Four patients did not show a sustained improvement; specifically, two patients with MM at histological examination underwent major surgery prior to salvage treatment thus resulting in persistent disability. According to our results it can be concluded that tumor involvement is not uncommon in MM patients diagnosed as

having ONJ. For this reason, at variance to what is recommended in published guidelines, in MM patients with ONJ, biopsy of the lesion should be performed, though with caution, in order to rule out tumor involvement and to set up the most appropriate treatment.

PO-157

FLOW CYTOMETRIC EVALUATION OF THE BONE MARROW PLASMA CELL CLONE IN PATIENTS WITH MULTIPLE MYELOMA, MONOCLONAL GAMMOPATHY OF UNCERTAIN SIGNIFICANCE AND POLYCLONAL PLASMACYTOSIS

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Multiparametric immunophenotyping of multiple myeloma (MM) and other plasma cell (PC) dyscrasias represents an attractive approach for both research purposes and clinical practice. In fact, differential diagnosis between MM, monoclonal gammopathy of uncertain significance (MGUS), and polyclonal plasmacytosis may be difficult in some cases, especially in those with not much bone marrow infiltration. Moreover, despite the widespread use, the different phenotype expression between smouldering MM (sMM) cases and those requiring treatment (crabMM) still remains a challenge. Immunophenotyping studies demonstrated that myelomatous plasma cells lack the B lymphoid-associated marker CD19 and may express cell surface antigens associated with other haematopoietic lineages such as NCAM (neural cell adhesion molecule)/CD56. In this study, the PCs were identified by their characteristic light scatter distribution and by a sequential gating strategy, assessing CD19, CD45, CD38 and CD56 reactivity. Thirty-one crabMM, 15 sMM, 30 MGUS and 9 polyclonal plasmacytosis (polyPL) were studied. A statistically significant higher percentage of CD19+ PC were demonstrated in polyPL cases as compared with MGUS group, while the lowest proportion of PCs expressing CD19 was accounted in both crabMM and sMM (Figure 1A). In contrast, a progressive increase of CD56+ PCs was found from polyPL to crabMM and sMM through an intermediate value observed in the MGUS group (Figure 1B). To better dissect the best cut-off values of the two different immunophenotypic profiles for discriminating among the different groups of PC dyscrasias, constructing receiver operating characteristic (ROC) curve analysis were performed. For the purpose of this evaluation, crabMM and sMM were collectively analysed. We determined 83% (AUC=0.7, p=0.005) and 6% (AUC=0.8, p<0.0001) as the best cut-off values of CD56+ and CD19+ PCs which discriminate between MM and MGUS cases, respectively. On the other hand, MGUS cases are distinguishable from polyPL ones for a cut-off values of CD19+ PC expression of 59% (AUC 0.8, p=0.006), while CD56 marker failed to distinguish between the two groups. On the basis of these results, CD56 expression, categorized by 83% cut-off value, correctly clustered all polyPL cases (very low CD56 expression), 24/30 (80%) of MGUS group and 28/46 (61%) of MM cases (very high CD56 expression). On the other hand, CD19 expression, categorized by 6% cut-off value, correctly predicted all polyPC cases (high CD19 expression), 24/30 (80%) of MGUS group and 33/46 (72%) of MM cases (low CD19 expression). Thus, we designed a score system based on the expression of a malignant phenotype. Specifically, a score of 1 was assigned to either high CD56 expression or a low CD19 expression by PC. The devised score system allowed us to split cases in 3 groups with a different PC phenotype: score 0 (CD56low/CD19high), score 1 (either CD56high or CD19low) and score 2 (CD56high/CD19low). In this cohort of cases, 100% of polyPL, 73% of MGUS, 20% of sMM and 16% of crabMM cases scored 0, respectively (Figure 1C). On the other hand, only 10% of MGUS and roughly half of both crabMM and sMM cases scored 2. Finally, 1/3 of MM and 1/6 of MGUS cases showed either CD56high or CD19low phenotype, respectively. In conclusion, our results indicate that flow cytometry analysis may clearly identify a bone marrow involvement with normal plasmacells. Also, the worst phenotype was demonstrated, as expected, more prominent in MM cases. Whether the presence of such a phenotype (score 2) in MGUS cases may have an impact in predicting a different clinical outcome should be evaluated.

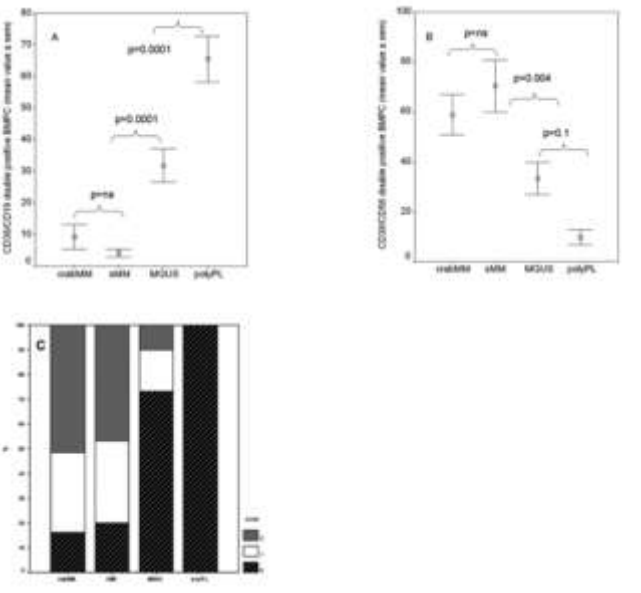

Figure.

PO-158

BONE DISEASE IN MULTIPLE MYELOMA PATIENTS CARRYING THE T (4;14) C HROMOSOMAL TRANSLOCATION

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It has been recently demonstrated that t(4;14) chromosomal abnormality is one of the most adverse prognostic factors predicting for poor outcome after high dose therapy and autologous stem cell transplantation in multiple myeloma (MM) patients. It has also been suggested that, at variance to what has been observed in other MM subtypes according to TC classification, bone involvement, as evaluated by spinal MRI, is relatively infrequent in these patients. In the present study we aimed at further testing this hypothesis by analyzing the extent of whole bone involvement in patients showing t(4;14) chromosomal translocation as compared to negative patients. For this purpose, 46 newly diagnosed MM patients (29M, 17F, median age = 54 yrs) underwent evaluation of total skeletal X-ray, whole spine MRI and, at the same time, quantification of markers of bone resorption (urinary NTX, PYR and DPYR and serum crosslaps) and bone formation (bone alkaline phosphatase-BAP and osteocalcin) was performed. Using a RT-PCR assay to detect the presence of IgH/MMSET fusion gene as a surrogate marker for t(4;14), we found 15 patients carrying this chromosomal abnormality, 7 of whom (46%) were also positive for the deletion of chromosome 13, this abnormality was detected in 11/31(35%) patients who proved negative for IgH/MMSET hybrid transcript. The two groups of patients did not differ significantly in terms of median age, distribution of M protein isotype and light chain, β-2 microglobulin, bone marrow plasma cell infiltration and disease stage. Spinal MRI was negative in 3/15 (20%) t(4;14) positive patients as compared to 13% t(4;14) negative patients; skeletal involvement, however, was more pronounced in t(4;14) positive patients (median skeletal score = 6.2, as compared to 3.58 in t(4;14) negative cases, p=0.00). These data were confirmed by the evaluation of bone resorption markers, as serum crosslaps were significantly increased in patients with t(4;14) abnormality compared to negative individuals (7972 pmol/L±1663SE vs 5133pmol/L±768SE p=0.04) Our results indicate that, despite a spinal involvement at MRI that is comparable to what is observed in negative patients, individuals who are t(4;14) positive show a more pronounced bone resorption pattern.

PO-159

EVALUATION OF BONE METABOLISM PARAMETERS AFTER ADMINISTRATION OF ZOLEDRONIC ACID AND BORTEZOMIB IN PATIENTS WITH MULTIPLE MYELOMA

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The observation of a number of parameters connected with bone metabolism, such as Piridinium derivatives, Telopeptide, Osteocalcin, Specific Alcaline Phosphatase, in patients with MM shows correlation between the bone reabsorption markers and disease activity, along with a tendency to normalization after therapy. Concerning bone formation indexes, the available literature reports inhomogenous data both at diagnosis and after therapy. An increase of alkaline phosphatase is sometimes observed following clinical response. The M.M. therapy is a combination of different drugs. It is therefore difficult to discriminate between the effects of byphosphonates on bone metabolism and those induced by chemotherapy. In a previous study we reported a decrease of renal excretion of piridinium derivatives in the early days following the administration of Zoledronic Acid. In the present study we asses the effect of Zoledronic Acid and Bortezomib (independently) on the excretion of piridinium derivatives and seric levels of osteocalcin. We measured these parameters in: a) 7 patients, 2 at diagnosis and 5 previously treated, before administration of Zoledronic Acid and in the following days, every 48 hours; b) 9 pts (who had not received Zoledronic Acid in the previous two months) during therapy with Bortezomib. 4 of them were treated for the first time, the other 5 had already received the drug. After Zoledronic Acid administration, renal excretion of piridinium derivatives tends to decrease, even if not in all the patients. On the contrary, the decrease of seric levels of osteocalcin is homogeneous and characterized by a mean value, compared to the initial value, equal to 82% after two days and to 80% after four days. After the first dose of Bortezomib renal excretion of piridinium derivatives does not seem to change significantly, but seric levels of osteocalcin decrease in all the patients but one, with a mean value of 63% compared to the one before therapy. The fast and direct effect of Zoledronic Acid on osteoclasts and on metabolites of bone reabsorption is well known. Bortezomib does not seem to exert a similar effect, but we plan to evaluate more specific and reliable markers such as telopeptide Both drugs seem to inhibit osteoblasts activity, at list in a first phase. It should be assessed if this activity is strengthened in case of combination therapy and if it persists during therapy, when the effects of Bortezomib on plasmacells become evident. We can suppose that the increase of PTH after bortezomib administration, reported in literature and observed in some of our patients, is due to the feed back subsequent to inhibition of osteoblasts.

PO-160

THALIDOMIDE-DEXAMETHASONE AND DOUBLE AUTOLOGOUS TRANSPLANTATION AS PRIMARY THERAPY FOR MULTIPLE MYELOMA: PROGNOSTIC IMPLICATIONS OF CHROMOSOME 13 ABNORMALITIES AND TRANSLOCATION T(4;14)

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A total of 142 patients with newly diagnosed and symptomatic multiple myeloma (MM) who were enrolled in the *Bologna 2002* clinical study of thalidomide-dexamethasone (thal-dex) and double autologous transplantation were investigated at baseline for the presence of chromosome 13 abnormalities [del(13)] and t(4;14). For this purpose the CD138+ plasma cell fraction was isolated from the bone marrow taken at diagnosis. Del(13) was analyzed by FISH, whereas t(4;14) was investigated using a validated RT-PCR assay designed to detect the presence of IgH-MMSET hybrid transcript. Overall, del(13) was present in 51% of patients, consistently with previous reports from the literature. Translocation t(4;14) was documented in 30% of cases; this value is higher than generally reported by FISH and reflects the higher sensitivity of RT-nested PCR assay. According to study design, patients received 4 months of front-line therapy with thal-dex and continued to receive thal-dex after stem cell collection, as well as between the first and second autologous transplantation with melphalan 200 mg/s.m. On an intent-to-treat basis, at least or higher partial response rate (according to Bladè criteria) to primary therapy with thal-dex was 81%, including 31% of patients in at least or higher very good partial response (VGPR). Strictly defined (immunofixation negative) complete remission (CR) rate following double autologous transplantation, whether it was actually received or not, was 54%. An analysis on an intent-to-treat basis revealed that 94 patients who carried both del (13) and t(4;14) at diagnosis had a significantly lower probability to attain at least or higher VGPR rate to primary therapy with thal-dex compared to patients with either del(13) alone (12% vs 41%, respectively; p=0.012) or t(4;14) alone (12% vs 50%, respectively; p=0.006). However, unfavourable response (attainment of at least or higher VGPR) to thal-dex conferred by the presence of both these chromosomal abnormalities was completely offset by double autologous transplantation. Indeed, on an intent-to-treat basis, at least or higher VGPR rate for patients with both del(13) and t(4;14) was 68% compared to 80% for patients with negative del(13) and t(4;14) (p=0.1). With a median follow-up of 24 months, the 3-year projected probabilities of overall survival (OS) and event-free survival (EFS) for all 142 patients were 80% and 59%, respectively (intent-to-treat). The presence or absence of t(4;14) had no significant impact on the 3-year projected probability of OS (80.12% vs 80.42%, respectively; p=0.3), whereas the presence of del(13) was of borderline significance (p=0.05). Consistently with these results, an analysis of patients who actually received double autologous transplantation and either carried or lacked both these chromosomal abnormalities showed that curves of OS and EFS were almost superimposable. Indeed, the 3-year projected probability of OS for patients with both del(13) and t(4;14) was 92% compared to 88% for patients with negative del(13) and t(4;14); (p=0.7), whereas the corresponding figures for EFS were 70% vs 77%, respectively (p=0.9). These results suggest that thal-dex combined with double autologous transplantation with melphalan 200 mg/s.m. may overcome the unfavourable prognosis conferred by del(13) and t(4;14). A longer follow-up is required before definite conclusions can be drawn.

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PO-161

EFFECTS OF ZOLEDRONIC ACID INFUSION ON CYTOKINES BEHAVIOUR IN MULTIPLE MYELOMA PATIENTS

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The main pathological feature underlying the bone disease in Multiple Myeloma (MM) is uncoupling of bone resorption from bone formation so that the resorption predominates. In the bone marrow (BM), MM plasma cells are in close contact with stromal elements and induce the relapse of several cytokines which modify the BM microenvironment, up regulating RANKL expression and secretion by both stroma and osteoblasts. Destruction of bone matrix induced by MM and its cellular interactions is accompanied by further release of cytokines. Biphosphonates are chemical compounds which selectively concentrate at the interface of the active osteoclasts and the bone resorption surface where they inhibit osteoclast activity. Recently, Santini *et al.* demonstred in solid tumors that zoledronic acid could have an *in vivo* antiangiogenic property through a significant and long-lasting reduction in serum VEGF levels. (Clinical Cancer Research, August 1, 2003 vol 9,2893-2897). Taking into account that angiogenesis play an important role in MM progression, we have investigated the behaviour of IL6, PDGF, IGF, TNFα and VEGF in a cohort of 29 (16 males and 13 females) consecutive MM patients with lytic bone lesions treated with 4 mg of zoledronic acid. Venous blood samples were drawn just before the beginning of drug infusion and at 1,2,7,21 (before the subsequent infusion) days after the zoledronic acid infusion. Serum samples were then tested for PDGF, VEGF, IL6, TNF α, and IGF-l with the R & D quantitative kits.(R & D Systems, Minneapolis, USA). Basal cytokine levels were compared with the values observed at 1, 2, 7 and 21 days using the Wilcoxon's test for nonparametric-dependent continuous variables. Differently from what observed by Santini *et al.* in solid tumors, serum VEGF median levels

did not decrease; on the contrary, it is significaly increased at days 7 after zoledronic acid infusion (P 0.0047). Moreover, IL 6 and TNFα significaly increased after 1 (p=0.0000 and p=0.0001 respectively) and 2 (p=0.0000 and p= 0.0021 respectively) days. Furthermore PDGF significantly decreased (p=0.005) after 2 days following zoledronic acid infusion. In conclusion, the cytokines' behaviour after the administration of zoledronic acid in MM is different from that observed in solid tumor; in particular, we did not observe a decrease in VEGF levels. This behaviour may partially explain why zoledronic acid did not affect MM progression as originally postulated.

PO-162

ANALYSIS OF RISK FACTORS FOR DEVELOPING BISPHOSPHONATE ASSOCIATED OSTEONECROSIS OF THE JAW AND EVALUATION OF ITS FREQUENCY IN MULTIPLE MYELOMA PATIENTS

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Background. Since 2003, reports have been published on osteonecrosis of the jaw (ONJ) possibly associated with the administration of bisphosphonates (BP). After the identification of 5 cases in our institution we decided to carry out a survey in other Italian centres. *Aims.* Aims of this study were to analyse the characteristics of all cases of osteonecrosis, independently from the primary disease (multiple myeloma (MM), solid tumors and other conditions); to research cofactors and to define the frequency of the event in multiple myeloma subgroup. *Matherial and methods.* We asked to the 69 GISL centres (Gruppo Italiano Studio Linfomi) to partecipate in a retrospective multicenter study on ONJ in cancer patients, filling out a form reporting ONJ and patients (pts) characteristics. To evaluate the frequency in MM we asked to determine the number of pts affected by MM treated with BP between 2002 and 2005. *Results.* 16 centres participated in the study and 14 observed cases of ONJ. BP related ONJ was identified in 35 pts. Females were 69%, and median age was 70 years old. All patients were affected by cancer (28 MM, 3 solid tumors, 3 myelodisplastic syndromes and 1 lymphoma). Median time from cancer diagnosis was 70 months. All patients were treated with BP: 14 received zoledronate, 3 pamidronate and 18 both drugs. Zoledronate was administered at standard dose of 4 mg, every 3-4 weeks; Pamidronate 60-90 mg every 4 weeks. Median duration of treatment with BP was 36 months (from 5 to 80 months) and 24 events manifested between 20 and 60 months Time for ONJ developing was shorter in pts treated with zoledronate in comparison with zoledronate plus pamidronate, and the difference is statistically significant (p<0.001). The 3 solid tumors were treated with chemotherapy and hormonotherapy. All MM pts had received one or more lines of treatment including, vincristine, doxorubicin and dexametasone (VAD), melphalan and prednisone (MP), stsroids and thalidomide alone or in combination, as well as high dose melphalan, as part of autologous bone marrow transplantation. ONJ involved mandible in 27 pts, maxilla in 6 and both in 2. In 18 pts out of 20 analysed, biopsy excluded cancer localization. Microbiological evaluation of the lesion was positive in 12 cases out of 15 performed, and in 8 cases Actynomices was found. In 18 patients ONJ was apparently spontaneous; in 16 it occurred after dental procedures. Parodonthopaties were reported in 17 pts. In 13 cases ONJ was complicated by fistulas, 7 by exposed bone or abscesses. BP were discontinued in 32 pts. Systemic antibiotic was administered in 33 cases; 8 pts underwent hyperbaric oxygen therapy and 20 surgical debridement. Several ps improved but only one was referred to be cured. *Conclusions*. our research confirms the association between ONJ and the use of BP. No definite risk factors could be identified with our retrospective analysis. However, considering our data, advanced age and female sex, steroids, anaemia, thalidomide in MM, and dental procedures/parodonthopaties could be suspected as additive risk factors. Although the frequency of ONJ is low, less than 2% in MM, it greatly reduces quality of life, and as no effective therapy is available, prevention is mandatory. Finally, we propose a pathobiologic model were we suppose that BP act on bone matrix and bone vascularization in association with other risk factors, in a complex multistep pathogenesis.

PO-163

VARIABILITY OF PHARMACOKINETIC OF THALIDOMIDE OVERTIME IN PATIENTS WITH MULTIPLE MYELOMA

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Thalidomide, as single-agent and in combination regimens, has shown strong activity in relapsed-refractory and newly diagnosed multiple myeloma (MM) patients. However, its mechanism of action, effects of the separate enantiomers and metabolites and dose- and concentration-effect relationships are not well known. Previous studies indicated the pharmacokinetic (PK) parameters were similar between healthy and patient's population. After a single dose of Thalidomide 200mg in healthy volunteers, absorption is slow, resulting in a peak concentration (Cmax) of 1-2mg/L at 3-4 hours (Tmax), with an apparent elimination half-life of 5-6 hours after administration. Although published articles on Talidomide are numerous, the pharmacokinetic in patients with MM are still ill defined. *Objective.* This study was conducted to evaluate the pharmacokinetic variability of Thalidomide overtime in patients with MM. *Materials and Methods.* Seven patients with MM at diagnosis, treated with Thalidomide (6 pts at the dose of 100 mg/day, 1 pt at the dose of 200 mg/day), were enrolled in this study. Serial plasma PK samples were collected at study entry (week 0) and at weeks 6 and 12 at pre-dose and at 1, 2, 3 and 4 hours after dosing. Plasma PK samples were assayed for Thalidomide concentration by validate HPLC-UV (High Performance Liquid Chromatography) assay. Plasma Thalidomide parameters were estimated: AUC0-4h, Ctrough, D/AUC0-4h, Cmax, 0-4h and Tmax, 0-4h during the 4 hours following drug administration. *Results.* Results are presented in the Table 1 (Pharmacokinetic parameters (Mean/SD) of Thalidomide). The mean inter- and intra-individual variability of systemic exposure was 64% and 74% respectively. *Conclusions.* The pharmacokinetic parameters of Thalidomide doesn't change overtime. However, the high intraindividual PK variability observed may be expected to have clinical significance. Further investigations are warranted to elucidate the PK processes involved.

Table 1. Pharmacokinetic parameters (Mean/SD) of Thalidomide.

Parameters	C_{trough}*	D/AUC_{0-4h}	$C_{max,\ 0-4h}$	$T_{max,\ 0-4h}$
Week 0	0.26/0.16	27.6/6.3	1.8/0.3	2.8/1.0
Week 6	0.30/0.26	28.3/21.1	2.4/1.5	2.9/0.4
Week 12	0.25/0.19	31.8/30.2	3.3/2.7	2.7/0.5
Significance	ns	ns	ns	ns

**Dose normalised*

PO-164

MULTIMODALITY IMAGING WITH 18F-FDG-PET/CT, 99MTC-MIBI AND MRI IN PATIENTS WITH MULTIPLE MYELOMA

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The aim of our study was to compare ^{18}F-FDG-PET/CT with 99 mTc-MIBI and MRI of spine and pelvis in the evaluation of patients with multiple myeloma, in order to assess the relative contribution of these imaging modalities in the staging of this neoplastic disease. *Materials and Methods.* Fifteen newly diagnosed patients with multiple myeloma were studied. Diagnosis and staging of patients were made according to standard criteria. All patients underwent whole-body ^{18}F-FDG-PET/CT, whole-body 99mTc-MIBI and MRI of spine and pelvis within ten days; the results of these imaging studies were compared. *Results.* ^{18}F-FDG-

PET/CT was positive in 14 patients (6 had focal uptake, 2 had diffuse uptake and 6 had focal and diffuse uptake), 99mTc-MIBI resulted positive in 12 patients (2 focal, 3 diffuse, 7 focal and diffuse) and MRI of spine and pelvis was positive in 12 patients (2 focal, 6 diffuse, 4 focal and diffuse). ^{18}F-FDG-PET/CT showed a total of 53 focal lesions (52 in bones and 1 in soft tissues) of which 38 in districts other than spine and pelvis whereas 99mTc-MIBI visualized 20 focal lesions (19 in bones and 1 in soft tissues) of which 16 in districts other than spine and pelvis. In spine and pelvis, ^{18}F-FDG-PET/CT detected 15 focal lesions (7 in spine and 8 in pelvis), 99mTc-MIBI visualized 4 focal lesions (all in pelvis) and MRI detected 21 focal lesions (13 in spine and 8 in pelvis). *Conclusion.* ^{18}F-FDG-PET/CT showed to be more sensitive than 99mTc-MIBI in the detection of focal lesions, although its specificity remains to be determined in a larger series of patients. ^{18}F-FDG-PET/CT and 99mTc-MIBI were more panoramic than MRI; however, MRI should be preferred for the detection of focal lesions in the spine.

PO-165

COMBINED ADMINISTRATION OF DARBEPOETIN AND ZOLEDRONIC ACID IN THE TREATMENT OF REFRACTORY MULTIPLE MYELOMA (MM)

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Background and Aims. Multiple myeloma (MM) is a neoplastic disease, that affects especially the elderly, even if in recent years it has also been observed in young patients. Painful osteolytic bone destruction is a frequent complication of MM. Bisphosphonate therapy has been shown to reduce complications of bone lesions in MM and in other malignancies. In details, it seems that zoledronate, a new generation aminobisphosphonate, also exerts antitumor effects on myeloma cells; this drug has cytotoxic activity because it causes apoptosis and block of the proliferation. Another frequent issue in patients with MM is anemia that is due both to disease progression and chemotherapy. Moreover, on the basis of the osteolytic bone lesions and anemia recorded in patients with MM, we will evaluate the percentage of regression of bone lesion after the treatment with zoledronate and the level of haemoglobin after the treatment with darbopoetin-α. It is well known that myeloma cells compete with normal progenitors, and above all with erythroid precursors, for the same marrow microenvironment. We hypothesize that the stimulation of erythroid precursors in addition to the concomitant anti-proliferative effects exerted by zoledronate on myeloma cells induce an anti-tumour effects in myeloma patients. *Methods.* Starting from June 2004, we are following 30 patients with stage II/III MM and 23 out of 30 are currently treated, independently from the adopted chemotherapy, with zoledronate (4 mg i.v. every 28 days) because they had osteolytic bone lesions at the diagnosis. In 8 out of 23 patients we have suspended chemotherapy after 12 cycles of Melphalan and Prednisone regimen for excessive toxicity even if they presented persistent disease at cytological examination of bone marrow blood and of serum markers. In these patients, on the basis of the anemia recorded (median Hb: 8.2 g/dL, r. 7.8-9.2), we have associated a treatment with 150 micrograms s.c. darbopoetin once a week. We have performed biological studies to evaluate the antiproliferative effect of zoledronate and darbopoetin, used alone and in combination, on myeloma cell lines. We have seeded myeloma cells in 96-multiwell plates ad after 24h incubation at 37°C the cells were incubated with 3 different concentrations of the drugs. We have based drug combination studies on concentration-effect curves generated as a plot of the fraction of surviving cells versus drug concentration, after 72h of treatment. *Results.* At 6 months from the beginning of the treatment with zoledronate all the 30 patients treated by zoledronate had a partial or complete regression of bone lesions. In the patients treated by darbepoietin, after 6 weeks, all the 8 patients had an increase of haemoglobin (median: +1.5 g/dL, r.: +1.2-2) and, at a clinical re-staging performed after three months from the beginning of Zoledronate-Darbepoietin combined administration, a partial remission was recorded in 6 out of 8 patients while the remaining was in persitent disease. The data obtained from biological studies show a clear antiproliferative effect of zoledronate and darbepoetin on myeloma cell lines, effect closely releated to drug concentration. *Conclusion.* In our study, the combined administration of zoledronate and darbepoetin in patients affected by refractory myeloma multiple likely induce the anti-tumor effects observed in these patients. That anti-proliferative effect seem to be confirmed from *in vitro* studies. Further studies are in progress in order to investigate the antiproliferative and anti-apoptotic effects of the combination on myeloma cells collected from patients.

PO-166

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

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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 proteasome inhibitors 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 as well as doxorubicin in solid tumor cells. Among the drugs that have been recently tested with anti-MM activity, arsenic trioxide (As_2O_3) is able to induce MM cell apoptosis and to inhibit cell growth. Preliminary clinical trials have been recently performed showing that As_2O_3 has anti MM effect even if resistance may occur. In this study we have investigated the potential *in vitro* effect of ZOL in combination with As_2O_3 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 lines JJN3 in combination with As_2O_3 at clinically achievable levels (0.5-2 μM). We evaluated MM cell proliferation with 3H-thymidine incorporation and MM cell apoptosis by flow cytometry checking both early and late apoptotic cells and by western blot to evaluate caspase activation. 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 inducing caspase-8 activation in combination with As_2O_3 in fresh purified CD138$^+$ MM cells. The capacity of ZOL to increase drug-induced MM cell apoptosis was also observed blocking the proteasome activity and NF-kB activation by PS-341. In fact we found that ZOL increased the pro-apoptotic effect of PS-341 (4-8 nanoM) in JJN3 and induced apoptosis of the PS-341 resistant cells DHL-4 in combination with PS-341. A further enhancement of cell apoptosis was observed with the addition of As_2O_3. In conclusion our data indicate that ZOL at high concentrations increases the apoptotic effect of As_2O_3 and may overcome drug-resistance in MM cells suggesting that ZOL could be used in combination with the new cytotoxic drugs in MM therapy.

PO-167

MAINTENANCE WITH VERY LOW DOSE THALIDOMIDE AFTER AUTO-SCT IN MULTIPLE MYELOMA: LOW TOXICITY AND IMPROVED OUTCOME

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High dose therapy with single or double transplantation (auto-SCT) has improved prognosis of multiple myeloma (MM). New drugs are promising in upfront therapy while the role of maintenance is still debated. Thalidomide (thal) is an active drug in the treatment of myeloma, and is been investigated as first line therapy. It could be useful in the control of minimal residual disease. We used thal as maintenance after autologous transplantation (single or double) and compare the outcome with other maintenance or none. From January 2001 to May 2006 28 patients (14 males and 14 females) with MM have been treated in our institution. Median age was 59 years (range 40-72). 13 were IgG, 8 IgA, 1 IgD, 4 light chains and 2 plasma-cell leukaemia. Treatment was 4 cycles of VAD regimen followed by auto-SCT. 9/28 performed double auto-SCT. 3 months after SCT, 14 patients (10 single and 4 double SCT) began thal 50 mg/die as maintenance therapy. 14 patients (9 single and 5 double SCT) received

IFN-γ (4/13), dexa (3/13) or no therapy (7/13). The 2 groups were regarding the type of myeloma: 7 IgG, 3 IgA, 3 light chains and 1 plasma-cell leukaemia in the thal group; 6 IgG, 5 IgA, 1 IgD, 1 light chains and 1 plasmacell leukaemia in the other. Response to SCT: 4 CR, 9 PR and 1 NR in the thal group; 7 CR, 6 PR and 1 NR in the other. Relapsed in the thal or no thal groups 5/14 (35%) and 8/14 (57%) patients respectively. Median follow up from the diagnosis was 33 months (range 7-151) for every group. After auto-SCT, in the thal group the median PFS were 44 months and OS were 71% projected at 50 months, in no thal group the median PFS and OS were 10 and 21 months respectively. The difference between the 2 groups is statistically significant for PFS (p: 0,001), and not significant for OS from diagnosis (p: 0,057) even if difference (70% vs. 15% projected a 100 months) appears clear. (Figure 1-2). Thal was administered for a median period of 12 months, being neurological toxicity the main reason of suspension in 3/14 patients (21%). Neurological toxicity grade I-III was present in 65% of patients, while haematological toxicity grade I occur in 55% of patients. In conclusion, in a small number of patients low dose thal as maintenance after auto-SCT resulted in an improved PFS and OS when compared with other or none maintenance, with acceptable toxicity.

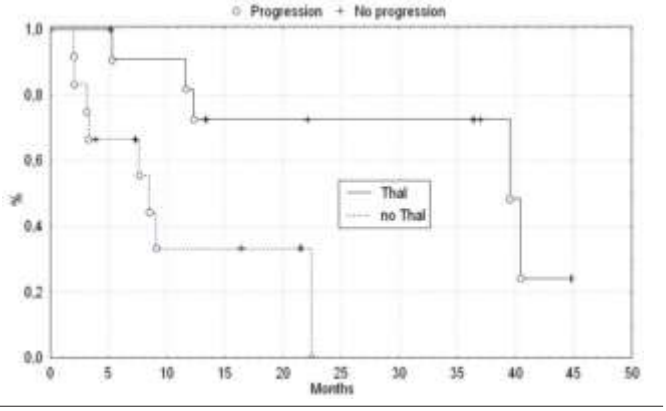

Figure 1. PFS post auto TMO in MM patients (Kaplan-Meier).

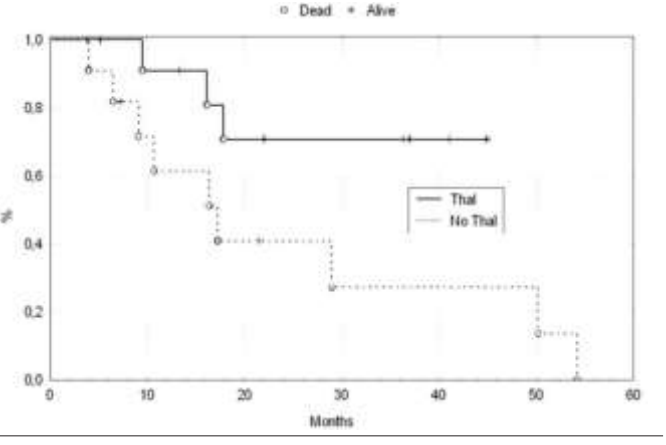

Figure 2. OS post auto TMO in MM patients (Kaplan-Meier).

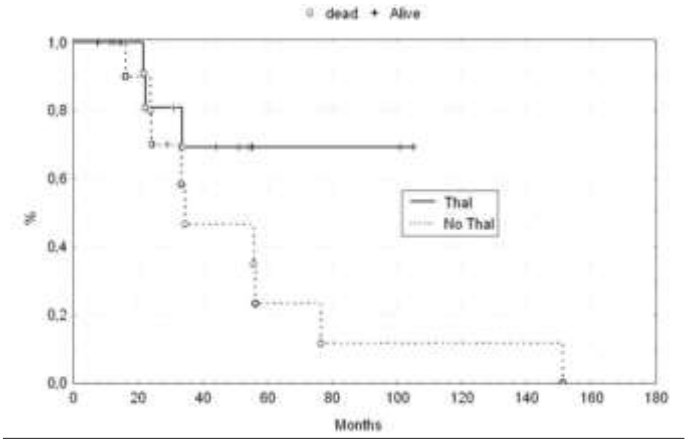

Figure 3. OS from diagnosis in MM patients (Kaplan-Meier).

PO-168

A PHASE I/II, MULTI-CENTER OPEN LABEL STUDY OF MELPHALAN, PREDNISONE, THALIDOMIDE AND DEFIBROTIDE IN AVANCED AND REFRACTORY MULTIPLE MYELOMA PATIENTS.

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Defibrotide (D) is a single-stranded polydeoxyribonucleotide which acts on the vascular endothelial cells. Usually employed in treatment and prophylaxis of veno-occlusive disease, D enhances factors that contribute to fibrinolysis and suppresses those that promote coagulation. *In vitro*, D had minimal inhibitory effect on MM or solid tumor cell growth; but *in vivo* (human MM xenografts in SCID/NOD mice), it showed activity as a single agent and increased the responsiveness of MM cells to cytotoxic chemotherapeutics, such as melphalan or cyclophosphamide. Thus, D may abrogate tumor cell interaction with bone marrow stromal cells and may overcome resistance of MM cells to conventional chemotherapy. At diagnosis, the combination of oral MP with thalidomide (MPT) increases the PR rate from 50% to 70% and the CR rate from 3% to 20%. Defibrotide might further improve the response rate of MPT. To address this issue safety and efficay of the association of Melphalan/ Prednisone/Thalidomide/Defibrotide (MPTD) as salvage treatment are being evaluated in a Phase I dose-finding study. Patients in first or second relapse, with a measurable desease are eligible for treatment. The MPTD regimen include: 24 patients receive six 35-days cycles of standard oral MP (Melphalan 0,25 mg/Kg daily and Prednisone 1,5 mg/Kg daily on days 1 to 4), Thalidomide 50 mg o.d. orally (p.o.) continuously. Defibrotide is administered at 3 different subsequent dose levels to evaluate the maximum tolerated dose. Each level consists of eight patients: the first 2 patients receive a fixed dose of Defibrotide intravenous (i.v.) during MP course and then Defibrotide orally until the end of cycle; the last 6 patients receive D orally during the entire cycle.

Level + 1 Defibrotide = 17 mg/Kg i.v. or 2.4 p.o. on days 1-4, followed by 1.6 g p.o. through day 35.
Level + 2 Defibrotide = 34 mg/Kg i.v. or 4.8 g p.o. on days 1-4, followed by 3.2 g p.o. through day 35.
Level + 3 Defibrotide = 51mg/Kg i.v. or 7.2 g p.o. on days 1-4, followed by 4.8 g p.o. through day 35.

At present, 8 patients have been enrolled: 6 patients were on Durie-Salmon stage IIIA, 2 patients on stage IIA, median B2-microglobulin was 3,9 mg/l, median Hb was 10.3 g/dL, median albumin was 3.4 g/dl, median bone marrow plasma cells were 65%. The treatment was generally well tolerated. The maximum tolerated dose of Defibrotide has not been reached yet. The most common grade I-II non-hematologic toxicities were: infections in 2 patients, fatigue in 1 patient, constipation

in 1 patient, tremors in 1 patient; somnolence in 2 patients, urinary incontinence in 2 patients, dizziness in 2 patients, hyperglycemia in 1 patients, dry skin in 1 patient. Four patients experienced grade I anemia; three patients grade II neutropenia, two patients grade I or II thrombocytopenia. One patient discontinued Thal during first cycle because of gastrointestinal toxicity (grade III) and then stopped treatment due to diagnosis of Amiloidosys. The study is currently ongoing and an update will be presented at the meeting.

PO-169

REMOVAL OF SERUM FREE LIGHT CHAINS BY HAEMODIALYSIS IN PATIENTS WITH MULTIPLE MYELOMA

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Renal failure caused by excess free light chain (FLC) production is common in multiple myeloma (MM), and is associated with poor outcome. There is interest in whether normalisation of serum FLC by plasma exchange (PE) and/or haemodialysis (HD) can improve renal outcomes. The purpose of this study was to demonstrate the ability of HD with a novel dialysis membrane to remove FLC in MM patients. Five MM patients with acute renal-failure underwent dialysis using a Gambro Protein Permeable HCO 1100 Polyamide membrane. Blood before and after each dialysis and samples of dialysate fluid were taken. FLC measurements were performed using the immunoassay FREELITE™. The total amount of FLC removed was calculated as follows: mean dialysate FLC concentration (mg/L) x dialysate volume (L). All 5 patients showed abnormal sFLC levels at presentation (3 with elevated serum free lambda; 2 with serum free kappa). The mean percentage falls of FLC were 59.6%, 58.6% and 23.7% for the lambda patients and 45.9% and 61.8% for the kappa patients (Table 1).

Table 1. Table of FLC levels in serum and dialysate fluid following dialysis.

Patient	*Dialysis Sessions*	*FLC type*	*Mean Blood Pre (mg/L)*	*Mean Blood Post (mg/L)*	*Mean% Removed*	*Total in Dialysate fluid (g)*
1	5	Lambda	10626	4310	59.6	33
2	2	Lambda	9155	3760	58.6	31
3	6	Lambda	3362	2445	23.7	8
4	10	Kappa	2758	1489	45.9	4
5	1	Kappa	861	329	61.8	19.6

The means of the total amount of FLC removed per dialysis session were 33 g, 31 g, 8g, 20 g and 4 g. We have conclusively demonstrated that HD can remove large quantities of FLC in the context of MM with the Gambro HC1100 dialysis membrane. The total amount of FLC found in the dialysate exceeded the available FLC in the blood, suggesting that FLC were also removed from the extravascular compartment. The use of extended HD could further improve serum FLC removal in these patients.

Chronic Myeloproliferative Disorders I

PO-170

THE CONCOMITANT OCCURRENCE OF WALDENSTROM MACROGLOBULINEMIA AND AMYOTROPHIC LATERAL SCLEROSIS

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Waldenstrom's macroglobulinemia (WM) is an indolent lymphoma characterized by the production of a monoclonal immunoglobulin M protein and several complications such as hyperviscosity, cytopenias and peripheral neuropathy. The presence of monoclonal macroglobulin autoreactive antibodies influences the clinical presentation and the natural history of the disease. A severe peripheral neuropathy constitutes one of the criteria for defining a disease progression and for initiating therapy in previously asymptomatic patients with WM. In these cases antibodies to peripheral nerve myelin (PNM) and antibodies to myelin associated glycoprotein (MAG) can be often but not always detected. Among neurological diseases the Amyotrophic Lateral Sclerosis (ALS), or other Motor Neuron Diseases (MND), have rarely been reported in association with Waldenstrom's Macroglobulinemia; it is postulated that such patients might have antibodies against motor neurons (for example anti GM1) which may cause motor dysfunction by binding to the peripheral nerve even if the pathogenesis of the disease is multifactorial. Some authors assessed that association of paraproteins with MND couldn't be only the result of a coincidence, but it is unclear whether WM play a role in the pathogenesis of ALS. We report the case of a patient affected by ALS associated with WM. In some months a 52-year-old previously healthy man had important weight loss (about 10 Kg in 3 months) together with progressive painless muscle weakness and wasting in all limbs, without sensory symptoms. He had fasciculation and cramping of muscles, especially in the feet, impairment of the use of the legs, tripping and difficult walking resulting in frequent falls, accompanied by pathologically brisk reflexes. The patient had difficulty in using the hands for the activities of daily living with progressive atrophy of the interosseous muscles of fingers. Other symptoms included thick speech, dysarthria. Electrodiagnostic tests including electomyography and nerve conduction velocity were consistent with Motor Neuron Disease. MRI didn't show any abnormalities of brain and spinal cord but there was an alterated signal intensity of bone marrow in the cervical, thoracic and lumbar vertebrae and the pelvis. A diagnosis of ALS was made; the patient started therapy with riluzole and α-tocopherol. Blood and urine tests were within normal ranges but a monoclonal IgM-k protein was detected by serum protein electrophoresis and confirmed by serum immuno-fixation (M immunoglobulin 1570 mg/dl, k chain 440 mg/dL). The bone marrow biopsy demonstrated a significant infiltration by small lymphoplasmacytoid cells, compatible with lymphoplasmacytic lymphoma/Waldenstrom's macroglobulinemia (LPL/WM). The bone marrow immunophenotype, which is variable in WM and overlaps with other B-cell lymphoproliferative disorders, in this case was as following: CD19+ CD5-, HLA-DR+, CD20+, CD22+, CD23-, FMC7+, CD19+ CD38+, k+, IgM+, IgD+. Bone marrow was not evaluable for cytogenetic study. Staging total body MRI didn't show linfoadenopathy nor organomegaly. Immunoblot studies on the patient's serum didn't revealed antibodies to MAG nor antibodies to HuD, Yo, Ri. We remember that antibodies to MAG, and often antibodies to sulfated glucuronic acid paragloboside (SGPG) are found in patients with sensorimotor peripheral neuropathy associated to monoclonal gammopathy but almost never in patients with ALS. The patient started treatment with the anti-CD20 monoclonal antibody rituximab, 375 mg/m^2 for 4 weekly infusions. Despite the improvement of MW, as revealed by the reduction of the monoclonal IgM protein to 484 mg/dL, the neurologic disease worsened as concerning in particular the dysarthria and difficult walking. This case report underlines the likely absence of a causal association between paraproteinemia of WM and ALS since an useful therapeutic approach for WM didn't show any efficacy in a rapidly evolving ALS.

PO-171

RELATIONSHIP BETWEEN JAK2V617F TYROSINE KINASE MUTATION AND ERYTHRO POIETIN-INDIPENDENT ERYTHROID COLONIES IN MYELOPROLIFERATIVE DISORDERS

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Background. Recently, a point mutation in the janus kinase 2 (JAK2V617F) has been described in the majority of patients with polycythemia vera (PV), as well as in subsets of patients with essential thrombocythemia (ET) and idiopathic myelofibrosis (IM). A constant hallmark of myeloproliferative disorders is an abnormal response to cytokines. The proliferation *in vitro* of erythroid progenitors in absence of erythropoietin (EPO) is a characteristic of PV patients. The precise role of JAK2 mutation in the pathogenesis of PV is still unknown even if both the hypersentivity to EPO of JAK2 mutant cells and the relationship between Jak2 mutation and endogenous erythroid colonies (EEC) have been demonstrated. There is however a group of PV that is JAK2V617F negative and it is unclear if it can be considered a distinctive myeloproliferative entity. The relationship between EEC, JAK2V617F and WHO diagnosis of PV is still matter of debate. *Aim of the work.* To study the relationships among diagnosis of PV/ET, EEC and JAK2V617F mutation. *Patients and methods.* Thirty five patients observed between June 2005 and April 2006 for erythrocytosis and/or thrombocytosis were considered for the present analysis. The initial diagnoses of PV and ET were retrospectively revised according to WHO criteria. All patients were bcr/abl negative. All patients were studied for both the presence of JAK2V617F mutation and the ability to form EEC. Peripheral blood granulocytes were prepared with the use of Ficoll gradient centrifugation. Granulocyte DNA was extracted with Salting out method. A quantitative real-time polymerase chain reaction (qRT-PCR)-based allelic discrimination assay was performed for the detection of Val617Phe JAK2 mutation. For the allelic discrimination of the Val617Phe JAK2 mutation a mixture of 1X SYBR PCR Master Mix, a single common forward primer and two reverse primer was performed. Granulocyte RNA was isolated with extrazol and Bcr/Abl transcript was determined. The *in vitro* assay for EEC was performed by plating 5×10^5 mononuclear cells in methocult H4531 medium in the absence of exogenous erythropoietin (EPO). Concurrent plates containing optimal amounts of EPO (2U/mlL were prepared as control cultures. Dishes were incubated in a humidified atmosphere of 5% CO_2 at 37 C° and erythroid colonies were scored at 14d by standard criteria. *Results.* Initial clinical diagnoses were secondary polyglobulia (SP), PV and ET in 21, 10 and 4 patients respectively. When diagnoses were retrospectively revised according to WHO criteria the four diagnoses of ET were confirmed, while only five of the ten PV diagnoses respected all WHO criteria for PV. The JAK2V617F mutation was found in only seven cases distributed as follows according to WHO criteria: four patients with PV, one with SP and one with ET. EEC were positive in eleven patients: the correspondence between JAK2V617F positivity and EEC was evident in six patients; one JAK2V617F positive case didn't form EEC; five JAK2V617F negative cases developed EEC. *Conclusions.* Our results on this small group of PV and ET patients show some discrepancies among the WHO diagnostic criteria, the JAK2V617F positive test and the ability to form EEC in order to define PV. An important group of five patients was positive for EEC, but negative for JAK2 mutation. Results are preliminary and a larger group of patients is needed, but a total overlap between the ability to form EEC and the Jak2 mutation is not evident.

PO-172

JAK2 (V617F) AS AN ACQUIRED SOMATIC MUTATION AND A SECONDARY GENETIC EVENT IN FAMILIAL MYELOPROLIFERATIVE DISORDERS

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Background. A somatic gain-of-function mutation of the Janus kinase 2 (JAK2) gene is found in Philadelphia (Ph)-negative chronic myeloproliferative disorders (CMD), which include polycythemia vera (PV), essential thrombocythemia (ET) and chronic idiopathic myelofibrosis (CIMF). The JAK2 (V617F) mutation occurs in a multipotent hematopoietic stem cell and produces a selective expansion of its myeloid-lineage cell progeny. Chronic myeloproliferative disorders appear to have a sporadic occurrence in most instances, but well-documented familial cases have been reported. *Aims.* The aim of this study was to define the biological and clinical significance of the JAK2 (V617F) mutation in familial chronic myeloproliferative disorders. *Patients and Methods.* Sixteen pedigrees with familial CMD were identified through family history investigation of apparently sporadic patients. Eleven families had homogeneous phenotypes (PV in 8 and ET in 3), while 5 had mixed phenotypes including PV, ET and CIMF. A quantitative real-time polymerase chain reaction (qRT-PCR)-based allelic discrimination assay was employed for the detection of the JAK2 (V617F) mutation. The X-chromosome inactivation pattern of clonality (XCIP) was established by the analysis of the IDS gene expression and by the analysis of DNA methylation at the HUMARA and PGK loci. *Results.* The main features of familial myeloproliferative disorders were indistinguishable form those of sporadic cases. JAK2 (V617F) was detected in granulocytes of 15/25 patients, but not in T lymphocytes. Granulocyte mutant alleles ranged from 5.3 to 91.5%, and increased with time in five patients. Distribution of mutant JAK2 within the same pedigree displayed an homogeneous pattern (5 families), or a discordant one (4 families). In female patients, the proportion of granulocytes carrying JAK2 (V617F) was lower than that of clonal granulocytes determined by analysis of XCIP. JAK2 (V617F) was not detected in granulocytes from 54 healthy relatives but a potential early PV was found in 2 of them. *Conclusions.* JAK2 (V617F) represents an acquired somatic mutation in familial cases as it does in sporadic cases, and likely occurs as a secondary genetic event on the background of pre-existing clonal hematopoiesis. This suggests that a genetic predisposition to acquisition of JAK2 (V617F) is inherited in families with myeloproliferative disorders, which might be less rare than thought. At the moment, we have under evaluation 4 additional families with CMD.

PO-173

EPHA3 TYROSINE KINASE RECEPTORS AS THERAPEUTIC TARGETS IN CHRONIC MYELOPROLIFERATIVE DISEASES.

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Background. Chronic myeloproliferative disorders (CMPDs) are hematological malignancies characterized by a clonal proliferation of one or several lineages. Although the recent advances in molecular biology and genomics the molecular mechanisms of Ph- CMPDs are still largely unknown. Activated tyrosine kinases and their cell-signal transduction pathways are to day of increasing interest also in the pathogenesis of Ph-CMPDs. Eph receptors tyrosine kinase and their ephrin ligands, are involved in many key developmental processes; their interaction triggers a bidirectional signals transduction cascade that regulates morphogenesis and cell-cell interaction. Although Ephs receptors are no detectable in normal adult tissues, they are overexpressed in many tumors, suggesting a possible role of these PTKs in oncogenesis. Dasatinib (BMS-354825), a novel BCR-ABL inhibitor exhibits an interesting inhibitory activity on some tyrosine kinases, such us EphA receptors. *Aim.* The aim of this study was to comprehensively evaluate the expression of EphA3 in CMPD and investigate the possibility of exploiting EphA3 as a therapeutic target of BMS-354825. Methods: EphA3 mRNA expression was analyzed, using Real Time PCR, in 266 samples obtained from CMPD patients (133 PB and 133 BM), 47 with a diagnosis of PV, 36 ET, 20 IM, 29 CMML, 4 HES, 31 CML in chronic phase and 70 patients with a diagnosis of Ph-CMPD. 38 normal controls (18 PB and 20 BM) were also evaluated. Moreover, we investigated the expression level of EphA3 in 34 sample of B-CLL, 50 AML, 22 ALL and in 7 cell lines (Jurkat, K562, HL-60, MEL, NIH-3T3, 293T, COS-7). Protein expression and localization were examined using Western Blot, Immunoprecipitation and Immunofluorescence analysis with appropriate antibodies. Transient transfection was performed in 293T e COS EphA3- cells using EphA3 plasmid. Nucleotide sequencing of tyrosine kinase catalytic domain was performed in 45 EphA3+ patients and in Jurkat cells. BMS-354825 incu-

bation of normal/pathological samples and cell lines was performed (3,10,20 nM). Cells proliferation was evaluated using MTT assay; apoptosis rate was analyzed by FACS (Annexin V) and colony growth was examined on methylcellulose culture. Results: We found EphA3 overexpression in Ph- mieloproliferative patients (40,3%) compared to normal controls; p=0,004 in the PB e p=0,005 in the BM. 17% of B-CLL, 32% of ALL, 26% of AML were positive but at significantly lower levels as compared to CMPDs. The overexpression was observed more frequently in BM as compared to PB (51,5% vs 22,8%). No expression difference was noted among the Ph-CMPD. Western Blot analysis confirmed protein expression in EphA3+ samples and revealed receptor phophorylation. Dasatinib led to significant dose-dependent inhibition of EphA3 phosphorylation. Moreover, BMS induced significant apoptosis (mean value 32,5%), colony growth reduction (mean value of 34,2 vs 76,5) and proliferation rate inhibition (48%) of EphA3+ cells compared to normal controls. Immunofluoresce assay showed transmembrane localization of EphA3 receptor and revealed cells projections reduction, cell repulsion and cell rounding only in EphA3+ transfected cells. No kinase domain mutations were found in EphA3 overexpressing patients and Jurkat cells studied. *Conclusion.* EphA3 is abnormally expressed in Ph-CMPD as compared to normal controls. EphA3 phosphorylation blocking induced by BMS-354825 results in growth arrest and apoptosis of EphA3 overexpressing cells. Therefore, EphA3 may represent a potential candidate for targeted signal transduction therapy in CMPDs.

PO-174

CD34+ CELLS OF IDIOPATHIC MYELOFIBROSIS DISPLAY REDUCED MEMBRANE EXPRESSION OF CXCR4 DUE TO GENE DOWN-REGULATION CELLULE CD34+ DI MIELOFIBROSI IDIOPATICA MOSTRANO UNA RIDOTTA ESPRESSIONE DI MEMBRANA DEL CXCR4 DOVUTA A DIMINUITA REGOLAZIONE DEL GENE

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Idiopathic myelofibrosis (IM) is characterized by constitutive mobilization of CD34+ cells in the circulation; this phenomenon distinguishes IM from the other Ph-negative chronic myeloproliferative disorders, as polycythemia vera (PV) and essential thrombocythemia (ET); additionally, CD34+ cell levels greater than 300×10^6/L have strong prediction value for disease evolution into acute leukemia (Barosi G *et al.,* 2001). The reasons for the abnormal release of CD34+ cells from the BM are not clear, and the hypothesis of simple physical dislodgment due to the presence of fibrosis seems unlike, as supported by the very low number of CD34+ cells that circulate in the peripheral blood of patients with marrow fibrosis secondary to pulmonary hypertension (Popat U *et al.,* 2006). Rather, it is suggested that the creation of a proteolytic environment, due to the release of proteases by the cellular progeny of neoplastic stem cells, would eventually facilitate CD34+ cell dislogment from the niches (Hoffman *et al.,* 2006). Thus, mechanisms involved in CD34+ cell release in IM would be at least in part similar to those in subjects receiving G-CSF for mobilization purposes, and acting on the SDF-1/CXCR4 axis. In this study, we have addressed the possibility that also cell-autonomous abnormalities are involved in the abnormal CD34+ cell circulation in IM by focusing on the receptor for SDF-1, CXCR4. We first observed that CD34+ cells from IM patients display significantly reduced CXCR4 membrane expression, as assayed by FACS analysis; Mean Fluorescence Intensity (MFI) was 5.2 and 1.6 on healthy control and IM PB-derived CD34+ cells, respectively (p=0.01). Interestingly enough, the CXCR4 expression on the PB CD34+ cells from either PV or ET patients did not differ significantly from controls. Also the percentage of CD34+ cells coexpressing CXCR4 was significantly reduced in IM patients (14%) as compared to normal subjects (54%, p=0.001) and PV (45%) or ET (38%) patients. By using quantitative Real Time PCR, the expression levels of CXCR4 mRNA in IM CD34+ cells were found significantly lower compared to normal BM-derived cells (median log10 RQ values were 0.0059 and 0.876, respectively; p<0.001). CXCR4 RNA levels were also significantly lower in the granulocytes from IM patients (median log10 RQ values were -0.23 vs 0.08 in the controls, p=0.14), while PV or ET granulocytes did not differ significantly from controls. These results indicate that the reduced expression of CXCR4 on IM CD34 cells is at least in part ascribable to reduced gene transcription. To address potential mechanisms, we evaluated the effects of CD34+ cell exposure to a range of SDF-1 concentrations up to 500 ng/mL; at 4 and 16 hours, the receptor membrane expression was dose-dependently reduced to almost undetectable levels, while the mRNA levels were unchanged, to suggest ligand-induced receptor internalization but no effect on gene transcription. Since a recent report (Kim HK *et al.,* 2006) indicates that exposure to G-CSF of myeloid cells results in lowering of membrane CXCR4 expression because of reduced gene transcription, we incubated CD34+ cells with G-CSF (200 ng/mL) for up to 16 hours; however, there was no modification in protein membrane content or CXCR4 mRNA levels. Similarly, TGF-β and IFN-γ did not induce appreciable changes in either protein or RNA content. Although the molecular basis for these observations need to be clarified, our results support an additional/independent mechanism for the constitutive mobilization of CD34+ cells in the circulation of IM patients, a part for proteolytic derangement of SDF1/CXCR4, that is represented by the reduced transcriptional activity of the CXCR4 gene.

PO-175

MICROVESSEL DENSITY AND EXPRESSION OF ANGIOGENIC CYTOKINES AND THEIR RECEPTORS IN AUTOIMMUNE MYELOFIBROSIS

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Autoimmune myelofibrosis (AM) is an emerging clinicopathological entity, that recognizes immunopathogenetic mechanisms and occurs isolately or in association with systemic and/or organ-specific autoimmune diseases. It is defined by a pattern including bone marrow, peripheral blood, serological and clinical features, and results in isolated or combined chronic peripheral blood cytopenias. It has to be distinguished from other disorders having myelofibrosis; among these, the most relevant differential diagnosis is with chronic idiopathic myelofibrosis (CIM), particularly when disclosing autoimmune clinical and/or laboratory features as epiphenomenon related to a secondary immune-dysregulation. AM is defined by increased reticulin fibrosis, not clustered megakaryocytes, reactive lymphoid infiltration in marrow biopsies, absence of significant tear-drop poikilocytosis and leukoerythroblastosis on peripheral blood smears, normal sized spleen, positive autoimmune serology. We purposed to contribute to the characterization of AM marrow changes through a comparative study with other diseases having myelofibrosis. In particular, we evaluated the microvessel density and the expression of angiogenic cytokines and their receptors in bone marrow biopsies derived from patients with AM or CIM, and from normal controls. Immunohistochemistry was performed by an immunoperoxidase method with avidin-biotin complex, using specific commercial antibodies (Santa Cruz Biotechnology, USA) on trephine biopsies derived, before treatment, from 8 patients with AM (6 females; median age: 65,8), 10 patients with CIM (7 females; median age: 65,4) in different disease phases, 10 normal controls (age and sex matching with the patients). Controls skipping primary antibodies were used as negative controls. Microvessel density, evaluated by immunostaining for CD34 antigen and counting of the number of vessels seen in 10 microscope fields (400X), resulted in a median value of 46.4 (range 9-112) in AM, whereas a median value of 180 (range 147-198) was estimated in the CIM biopsies. In addition, various isoforms of angiogenic cytokines and their receptors, including platelet derived growth factor (PDGF, PDGF A), basic fibroblast growth factor (bFGF) and its receptors (FGFR1, FGFR2, FGFR3, FGFR4), vessel endothelial growth factor (VEGF) and its receptor (VEGFR1), transforming growth factor β (TGFβ1, TGFβ2, TGFβ3) and its receptors (TGFβR1, TGFβR2), were evaluated for staining intensity and cellular distribution. Significant differences were detected in the expression of TGFβR1 on endothelial cells of small vessels as well as of bFGF on megakaryocytes, that resulted markedly decreased in AM compared to CIM. Our findings suggest that the assessment of microvessel density as well as the expression of some angiogenic cytokines and their receptors could be useful to differentiate AM from CIM.

PO-176

SIMULTANEOUS SCREENING FOR BCR-ABL REARRANGEMENTS AND JAK2 (V617F) MUTATION BY MULTIPLE RT-PCR AND CAPILLARY ELECTROPHORESIS IN CHRONIC MYELOPROLIFERATIVE DISORDERS

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Background. Classic chronic myeloproliferative diseases (CMDs) are a group of heterogeneous disorders including chronic myeloid leukemia (CML), essential thrombocythemia (ET), polycytemia vera (PV) and myelofibrosis with myeloid metaplasia (MMM). Because of frequent proliferation of more than one myeloid cell lineage, the diagnosis of the specific CMD type may be difficult although it may be considerably facilitated by the identification of genetic markers. The identification of genetic alterations involved in CMDs has provided relevant markers for diagnosis of these disorders as well as the basis for tailored therapy. For example, the reciprocal translocation t(9;22) leading to the BCR-ABL chimeric gene is the diagnostic hallmark of CML and its detection allows to assign patients to receive specific treatment with tyrosine kinase inhibitors. In 2005, several groups described a novel JAK2 point mutation (V617F, hereby referred to as JAK2mut) in classic BCR-ABL negative CMDs. This genetic alteration is detected in about 90%, 50% and 30% of PV, IMF and ET patients, respectively. The identification of the JAK2 mutation unambiguously establishes the presence of a clonal disorder and represents an additional relevant molecular marker which allows better diagnostic refinement in patients with CMDs. *Methods.* We describe here a multiplex RT-PCR assay with fluorochrome primers followed by capillary electrophoresis, designed to simultaneously screening for the two main genetic lesions associated with CMDs, i.e the BCR-ABL fusion relevant to confirm or exclude CML, and the JAK2 V617F mutation (JAK2mut) that characterizes PV and a high proportion of ET and MMM cases. The assay was applied to 50 CMD patients whose differential diagnosis was uncertain due to elevation of β2 myeloid cell types in their total blood counts at presentation. *Results.* JAK2 amplification fragments of 364 bp (blue, internal control) and 203 bp (blue, JAK2mut allele specific product), and BCR-ABL fragments (green) were amplified simultaneously in serial dilution experiments with a sensitivity of 1%. Based on the results obtained in the 50 cases screened, patients were divided in 3 groups as follows: JAK2 negative (JAK2wt)/BCR-ABL positive (9 cases, group 1), JAK2mut/BCR-ABL negative (35 cases, group 2), and JAK2wt/BCR-ABL negative (6 cases, group 3). Patients in group 1 were diagnosed as having CML and disclosed the b3a2 and the b2a2 fusion in 5 and 4 cases, respectively. Final diagnosis for patients in groups 2 and 3 was established according to the WHO criteria in combination with the molecular results. In particular, 8 patients in group 2 were diagnosed as having PV vera, 4 as MMM and 23 as ET. Finally, a diagnosis of ET and MMM was made in 5 and 1 patients in group 3, respectively Our approach allowed rapid diagnostic refinement in 44 cases including 9 CML patients who tested BCR-ABL+ve/JAK2wt, and 8 PV, 23 ET and 4 MMM cases who all tested BCR-ABL–ve/JAK2 mut. *Discussion.* In this study, we show that a multiplex RT-PCR assay followed by capillary electrophoresis can be used for simultaneous screening of the BCR-ABL fusion gene and JAK2 (V617F) mutation, i.e. the two most common genetic markers of CMDs. We believe that our approach may simplify and speed diagnostic refinement in this heterogeneous clinical context. In fact, this single-tube reaction permits on the one hand rapid identification of either genetic alterations. Moreover, compared to conventional PCR and agarose gel electrophoresis, the use of primers with distinct fluorochromes combined to capillary electrophoresis allows better distinction of amplification products of similar size, such as the b2a2 and JAK2 internal control. Besides the cases with overlapping laboratory findings described in the present study, we believe that our assay can be usefully adopted as a routine screening test for thrombocytosis of unknown origin. In these latter cases, the combined double marker approach would permit to confirm or rule out CML presenting with isolated thrombocytosis, as well as to confirm in cases carrying the JAK2mut, the presence of clonal expansion within a CMD. In conclusion, we suggest that this rapid, highly reproducible and nonisotopic assay amenable to automation, can be conveniently utilized in routine genetic diagnosis of CMDs. In addition to contributing the differential diagnosis among the distinct types of CMDs, the assay may be helpful in ruling out reactive conditions mimicking certain CMDs such as secondary thrombocytosis and erythrocytosis.

PO-177

SURVIVIN EXPRESSION, APOPTOSIS AND PROLIFERATION IN CHRONIC MYELOMONOCYTIC LEUKEMIA

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Chronic myelomonocytic leukemia (CMML) is a clonal disorder of a bone marrow stem cell characterized by the association of myelodysplastic and myeloproliferative features. Abnormalities in the regulation of the myeloid pathways for cellular proliferation, maturation and survival are the most important pathophysiologic mechanisms, but no specific genetic defects have been identified so far. We analyzed the expression of survivin, an inhibitor of apoptosis of the IAP gene family, in bone marrow cells from patients with CMML to evaluate possible differences in comparison with normal controls, and with other myelodysplastic (MDS) and myeloproliferative syndromes, and to investigate a possible correlation between survivin expression and altered apoptosis, as measured by TUNEL technique, or proliferation, as evaluated by MIB-1 immunostaining. Moreover, we evaluated whether abnormalities in survivin expression were associated with relevant laboratory and clinical findings. Survivin was detected by an immunoalkaline phosphatase method using a primary murine monoclonal antibody raised against human recombinant survivin (clone 8E2, NeoMarkers) on bone marrow smears from 34 patients with CMML (18 MDS-CMML and 16 MPD-CMML), 90 patients with MDS (34 RA, 17 RARS, 27 RAEB and 12 RAEB-t), 41 patients with AML, 19 patients with chronic myeloproliferative disorders (MPD) and 25 non hemopathic subjects. In normal samples survivin was never detectable. In CMML survivin levels higher (median 23.5%, IQR 16-33.5%) than in MDS (median 8%, IQR 5-15%) (p<0.0001) and AML (median 14.5%, IQR 3-23.5%) (p=0.01), but similar to those found in MPD (median 15%, IQR 10-33%) were observed. In CMML and MDS apoptosis was significantly higher compared to normal controls and all other subtypes of leukemias (p<0.0001). Proliferation did not differ significantly in normal controls, MDS and CMML; the lowest levels were observed in AML and MPD (p<0.0001). In CMML there was no correlation between survivin expression and blast cell percentage, apoptosis or proliferation, FAB or WHO subgroup. Proliferation was higher in the MDS-CMML subtype and tended to correlate with overall survival. In the subgroup with 10% or more bone marrow blasts cases with higher survivin expression showed higher evolution rate and shorter survival. In conclusion, CMML, like MDS but differently from MPD, is a disorder characterized by high proliferation and apoptosis. Survivin overexpression, by disrupting the balance between cell proliferation/differentiation and apoptosis, may play an important role in its pathophysiology. The detection of survivin-deregulated expression may provide a useful tool for diagnosis, prognosis and a possible target for experimental treatments.

PO-178

PS-341 (BORTEZOMIB) INHIBITS PROLIFERATION IN MO7E CELL LINE: AN INTERESTING *IN VITRO* MODEL FOR IDIOPATHIC MYELOFIBROSIS

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Idiophatic myelofibrosis (IMF) is a hematological disorder characterized by fibrosis, hypercellularity, excessive deposits of extracellular matrix proteins, and neoangiogenesis in the bone marrow. Recently, a spontaneous NF-kB activation in megakaryocytes from patient with IMF has been reported, with consequent increased TGF secretion. On this basis, inhibitors of the ubiquitin-proteasome pathway could be tested in the IMF. Bortezomib resulted effective as anti-proliferative agent in many tumour cell lines and neoplasias. On these bases, we evaluated the *in vitro* cytotoxic activity of this proteasome inhibitor on a megakaryoblastic cell line (MO7-e), in addition to other acute leukemia cell lines.

To evaluate the effect of Bortezomib on cell growth, proliferation assays were performed on MO7e, HL-60, HL-60/DNR, K562/R7, KG1a cell lines, using increasing drug concentrations (from 2.5 to 20 nM). For this test, cultured cells were exposed to Bortezomib for 12, 24, 48 and 72 hours. Bortezomib significantly reduced proliferation of MO7-e cell line, in a dose- and time-dependent manner: a significant anti-proliferative activity was observed as early as 24h after treatment and lasted for 72h. The dose-response curve showed the IC50 to be between 15 and 20 nM. On the contrary, Bortezomib did not affect proliferation rate of HL-60, HL-60/DNR, K562/R7, KG1a cell lines. Apoptosis was evaluated by morphological examination and by Annexin-V and caspase-3 staining; not significant differences in apoptosis rate between untreated and treated cells were observed. Because Bortezomib has been reported to enhance ROS generation, experiments were performed to address this issue. Nevertheless, Bortezomib did not significantly enhance ROS production in Mo7-e. Analogously, a not significant differentiating effect was noted in cells treated with Bortezomib for 72h or 6 days in comparison to untreated counterpart. In summary, this study, exploring the antitumor activity of bortezomib in several acute leukemia cell lines, support evidence indicating that this agent, with its anti-proliferative lineage-specific effect, may be useful as potential therapeutic agent for IMF, a myeloproliferative disorder still now lacking an effective cure.

PO-179

REDUCED CD26 DIPEPTIDYLPEPTIDASE IV MEMBRANE EXPRESSION AND ACTIVITY OF CD34+ CELLS FROM PATIENTS WITH MYELOFIBROSIS WITH MYELOID METAPLASIA

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Myelofibrosis with Myeloid Metaplasia (MMM) is characterized by an abnormal constitutive mobilization of CD34+ stem/progenitor cells and by SDF-1a (stromal-cell derived factor 1 α) plasma levels significantly higher than in patients with Policytemia Vera, Essential Thrombocytemia (PV/ET) and in healthy subjects (CTRLs). The SDF-1α axis chemoactracts hematopoietic stem and progenitor cells and is thought to play a crucial role in the homing/mobilization of these cells from the bone marrow, interacting with its unique receptor CXCR4. CD26 (dipeptidylpeptidase IV/DPPIV) is an extracellular peptidase, present both as membrane-bound and as a catalitically active soluble form in plasma. It has the ability to cleave SDF-1α at its position-2 proline inducing significant changes in its receptor binding and/or functional activation, thus inhibiting normal SDF-1α induced migration. In this study we evaluated the expression of CD26/DPPIV in patients with MMM (n=63) and in CTRLs (n=7) and its functional activity (MMM n=8; CTRLs n=3). CD26/DPPIV cell surface expression was measured by flow cytometry on peripheral blood mononuclear cells (PBMNC) or on immunoselected circulating CD34 pos cells. The CD26/DPPIV activity was measured in 96-well microplates using the chromogenic substrate gly-pro-p-nitoanilide. The proteolytic activity was determined by measurements of the amount of nitoanilide (pNA) formed in the supernatant at 405 nm. Absorbance was measured at 405 nm and the picomoles of pNA formed were calculated by comparison with a pNA standard curve. The CD26 expression on the membrane of PBMNC of patients with MMM was comparable to that found on PBMNC of CTRLs (median 29.5%, range 0.72-61.1; median 33.1%, range 20.3-39.2, respectively). However, the percentage of circulating CD34+CD26+ cells was statistically different ($p<0.05$) between patients with MMM (median 0.2%, range 0-48.4) and CTRLs (median 10.3%, range 0-30.2). The CD26/DPPIV activity evaluated on purified circulating CD34+ cells from patients with MMM was significantly lower ($p<0.03$) than that of CD34+ cells from CTRLs (median 14.2 pmol, range 0-45.6; median 67.2 pmol, range 34.5-81.1, respectively). The same activity, evaluated on CD34 neg circulating cells was comparable between patients with MMM and CTRLs. In addition, the peptidase activity mediated by the sCD26 was significantly lower ($p<0.03$) in patients with MMM (median value of absorbance 409, range 281-681) than in CTRLs (median 518, range 494-550). In summary, our data show that both CD26 expression and its peptidase activity are lower in circulating CD34+ cells of patients with MMM than in CD34+ cells of CTRLs; similarly, a significant reduction of the activity of sCD26 peptidase, derived from all the CD26+ peripheral blood cells, was found in patients with MMM compared with CTRLs. These novel findings shed some new light on the complex mechanism underlying the altered CD34+ cell mobilization of patients with MMM, suggesting that not only modifications of CXCR4 expression but also an altered regulation of SDF-1 activity can affect the correct function of the CXCR4/SDF-1 axis in patients with MMM.

PO-180

THE V617F JAK2 MUTATION INCIDENCE IN CHRONIC MYELOFERATIVE DISORDERS

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Chronic myeloproliferative disorders (cMPD) include several pathologies sharing the common feature of being clonal hematopoietic stem cell diseases. Based on the knowledge of the pathogenesis of chronic myeloid leukaemia, which involves the dysregulation of Abl kinase, it was speculated that another constitutively activated tyrosine kinase could account for the MPDs. Recently, recurrent and activating G to T point mutation resulting in substitution of phenylalanine for valine at position 617 (V617F) in the Janus kinase 2 (Jak2) was reported in bcr/abl-negative cMPD, including polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF). Estimates using different detection methods indicate that JAK2V617F is present in 65%-97% of PV, 23%-57% of ET, and 35%-57% of IMF patients, respectively. However, the distribution pattern among cMPD is changing since more sensitive methods, other than time consuming and not always feasible sequencing technique, were developed. In this study, we used an allele-specific polymerase chain reaction (PCR) assay (designated as the AS-PCR assay) to detect JAK2V617F. Briefly, a mutation-specific forward primer containing a fluorescent tag (FAM) was used in a PCR reaction with a wild-type sequence reverse primer. Only mutated DNA will be amplified (203bp), if present, and the PCR reaction product was analyzed using capillary electrophoresis in an automated genetic analyzer. To evaluate the sensitivity of the technique employed, either bone marrow (BM) or peripheral blood (PB) were analyzed, while the background signal was examined by using 50 normal PB samples. All normal samples resulted negative, showing a median signal one log lower than cases belonging to positive PB samples obtained from cMPD. BM JAK2V617F mutated cMPD cases sowed a fairly expected two logs higher signal than normal controls. In this study, we analyzed 29 PV, 24 TE, 21 MIF, 7 bcr/abl cMPD and 24 secondary polyglobulia (secPoly). Out of 105 cases analysed, 68 resulted positive. In particular, none of secPoly cases resulted muted, while 96.6%, 75%, 76.2% and 85.6% showed JAK2V617F mutation in PV, TE, MIF and cMPD, respectively. In conclusion, it is thus conceivable that the detection of JAK2 V617F will be used as an initial tool in the diagnosis of chronic hyperleukocytosis, thrombocytosis and erythrocytosis. Clinical and laboratory differences, if any, among MPDs that carry JAK2 V617F with those showing a wild type gene expression, especially in terms of bone marrow histology, will be presented.

Chronic Myeloproliferative Disorders II

PO-181

PROGNOSTIC SIGNIFICANCE OF THE ANGIOGENIC GROWTH FACTORS IN MYELOPROLIFERATIVE SYNDROMES

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The role of angiogenesis in the progressive growth and metastatic process of tumours is well established, but the clinical prognostic significance of the angiogenic factors in malignant haematological diseases is still not clear. In this study, we have assayed serum levels of two major angiogenic factors, the vascular endothelial growth factor (VEGF) and the basic fibroblast growth factor (b-FGF), in 55 patients affected by chronic myeloproliferative disorders (CMD). 25 of them were affected by essential thrombocythemia (ET), 10 by chronic myelocytic leukaemia (CML), 14 by polycythemia vera (PV) and 6 by primary myelofibrosis (MF). These patients were compared to 55 healthy sex- age-matched subjects (control group). In all patients the VEGF concentration was significantly increased respect to the control group ($p<0.01$). The highest concentration of VEGF, between the four groups of patients, was found in the subjects with ET (178.25±125.22 pg/mL, mean ± Standard Deviation). Moreover, the VEGF levels were significantly higher in CMD patients with vascular complications than in CMD without complications ($p<0.01$). The b-FGF levels also appeared to be significantly higher in the CMD patients compared to the control group ($p<0.05$). A significant correlation was found between the VEGF levels and the platelet count in the ET patients and the spleen index in the CML patients. VEGF level, in this study, is associated with increased risk of thrombotic complications. In conclusion, we can affirm that there is an evidence of increased levels of soluble angiogenic factors in malignant haematological disorders, therefore a high level of VEGF and/or b-FGF is a negative prognostic finding in this kind of pathologies.

PO-182

EXTRAMEDULLARY MANIFESTATION OF MYELOPROLIFERATIVE DISORDER WITH THE 5'KIAA1509/3'PDGFRB FUSION GENE

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The myeloproliferative disorders (MPDs) are characterized by the abnormal proliferation of one or more myeloid cell types. Unlike the Philadelphia chromosome in chronic myeloid leukaemia, there is no specific chromosomal abnormality associated with the MPDs. However, a number of recurrent chromosomal rearrangements, involving a variety of tyrosine kinase genes as PDGFRA, PDGFRB, FGFR1, and JAK2, have been reported. In this report, we describe the first MPD patient showing a t(5;14)(q32;q32) rearrangement in bone marrow and in extramedullary site of disease. As a consequence of this rearrangement, the 5' region of the KIAA1509 gene was fused to the 3' portion of PDGFRB. We performed a molecular cytogenetic analysis by FISH with specific BAC clones to identify the genes mapping in correspondence to chromosomal breakpoints. The use of RP11-368B7 and RP11-754J8 probes revealed the involvement of the KIAA1509 and PDGFRB genes in the reciprocal translocation. RT-PCR experiment using a KIAA1509 exon 11 forward primer (KIAA1509-11F) and a PDGFRB exon 11 reverse primer (PDGFRB-11R) have been carried out to reveal the precence of the 5'KIAA1509/3'PDGFRB fusion transcript; an amplification product of about 200 bp was obtained. The sequence analysis demonstrated that breakpoints were located within KIAA1509 intron 11 and PDGFRB intron 10. The fusion protein domains were identified using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). This chimeric protein was composed of 2 N-terminal KIAA1509 domains (coiled-coil myosin heavy chain tail and chromosome segregation ATPases region) and a C-terminal PDGFRB domain (catalytic tyrosine kinase). The use of different primers combinations revealed the absence of the reciprocal 5' PDGFRB/3'KIAA1509 fusion transcript. Further molecular studies on genomic DNA were performed to exactly define the breakpoints location within the KIAA1509 and PDGFRB genes. PCR experiment with the PDGFRB-100R and KIAA1509-100F primers generated a 100 bp amplification product. Sequence analysis of the breakpoint region has been carried out. The same molecular rearrangement was detected in genomic DNA sample obtained from a gastric lesion observed in the patient. The patient, treated with low-dose imatinib, achieved hematological and gastric lesion remission, without obtaining the molecular response. In this study we report the first case of Ph negative MPD in which a molecular marker was detected in an extramedullary site of the disease. Our patient showed a t(5;14)(q32;q32) producing a 5' KIAA1509/3' PDGFRB fusion gene. Only one case with a t(5;14)(q32;q32) was previously reported in literature. In our patient the KIAA1509 breakpoint was mapped within intron 11 instead of intron 9; any difference was observed in PDGFRB breakpoint location. As a consequence of this diversity, a larger fusion protein was produced including an additional chromosome segregation ATPases domain. Further studies are needed to elucidate the role of this fusion gene in the pathogenesis of myeloproliferative disorders.

PO-183

REDUCED EXPRESSION OF CXCR4 ON CIRCULATING CD34+ CELLS OF PATIENTS WITH MYELOFIBROSIS WITH MYELOID METAPLASIA (MMM) IS ASSOCIATED WITH HEMATOPOIETIC PROGENITOR CELLS MOBILIZATION AND ADVANCED DISEASE PHENOTYPE

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MMM is a clonal Philadelphia-negative chronic myeloproliferative disorder characterized by the constitutive release from the bone marrow of hematopoietic progenitor cells (HPCs) and by their accumulation in the blood and extramedullary sites. The mobilization of HPCs in humans is regulated by a network of cytokines, chemokines and adhesion molecules, among which stromal derived factor-1α (SDF-1a) and its unique receptor CXCR4 play a major role. The aim of this study was to investigate the CXCR4 expression on CD34+ cells of patients with MMM and compared it with CD34+ cells of patients with other Ph-negative myeloproliferative disorders (Ph-CMPD) and healthy subjects (CTRLs). We studied 72 patients with MMM, 8 with other Ph-CMPDs and 11 CTRLs. By cytofluorimetric analysis we evaluated on electronically gated circulating CD34+ cells both the membrane and the intracellular expression of CXCR4 both as percentage and mean fluorescence intensity (MFI). Plasma levels of SDF-1a were evaluated by ELISA. The expression level of CXCR4 gene was determined by quantitative Real-Time PCR using the Pre-Developed TaqMan Assay Reagent from Applied Biosystems concurrently with the housekeeping GAPDH gene. The percentage of CD34+CXCR4+ was significantly lower ($p<0.001$) in MMM patients (mean 41.6%; range 0.63-93.2) compared to CTRLs (mean 70.2%; range 37-97) or patients with other Ph-CMPDs (mean 63.5%). The CXCR4 MFI of CD34+ cells was also significantly lower ($p=0.003$) in MMM patients (mean 1.45; range 0.02-7.05) compared to CTRLs (mean 3.50; range 0.37-24.1) or patients with other Ph-CMPDs (mean 2.21; $p=0.02$). However, both the intracellular percentage and the MFI of CD34+CXCR4+ cells in patients with MMM were comparable to that of CTRLs (mean 6.48; mean 12.4, respectively). In 23 MMM patients the mRNA levels of CXCR4 in purified peripheral blood CD34+ cells were measured and compared to those obtained in CD34+ cells purified from 7 CTRLs. The mean (±SD) δCt value for MMM CD34+ cells (5.12±2.42) was significantly lower ($p<0.05$) than for CD34+ cells purified from normal CD34+ cells (mean 6.53±0.79). Plasma levels of SDF-1a in MMM patients (median 4160 pg/ml; range 1560-14875 pg/ml) were higher ($p<0.05$) than in CTRLs (median 2046 pg/ml; range <1560-8919); no statistical correlation was found between the percentage of CD34+CXCR4+ cells and SDF-1a plasma levels. On the contrary, we found an inverse correlation (R=-0.55; $p=0.005$) between CXCR4 expres-

sion on circulating CD34+ cells and the number of circulating CD34+CD45+ cells hematopoietic progenitor cells. Moreover, CXCR4 down-regulation was correlated with decreasing Hb concentration (R=0.384; p=0.001), older age (R=-0.31; p=0.026), longer disease duration (R=-0.37; p=0.008), and lower platelet count (r=0.42; p=0.007). In summary, we have documented in patients with MMM a membrane reduced expression of CXCR4, which is associated with a down-regulation of the expression of the gene. We have also shown that these findings are associated with disease characteristics that have an important clinical and prognostic relevance. We conclude that the signaling defect of CXCR4 we observed in MMM patients identifies a new biological characteristic of the disease that distinguishes MMM from the other Ph-CMPD.

PO-184

THE V617F JAK2 MUTATION IS PREDICTED BY THE HEMATOCRIT LEVEL AND BY PLATELET AND WHITE CELL COUNTS IN PATIENTS WITH ERYTHROCYTOSIS

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The Polycythemia Vera Study Group (PVSG) first developed a set of accurate criteria for the diagnosis of polycythemia vera (PV). Recently, different groups reported the existence of an activating mutation of Janus kinase 2 (Jak2) in many patients with one of the classic myeloproliferative disorders. Using different detection methods, the recurrent and activating G to T point mutation resulting in substitution of phenylalanine for valine at position 617 (V617F)in the JAK2 was estimated to be present in 65%-97% of PV cases. For this reason, the detection of JAK2V617F was incorporated in PV diagnostic criteria. In this study, we used an allele-specific polymerase chain reaction (PCR) assay (designated as the AS-PCR assay) to detect JAK2V617F. Briefly, a mutation-specific forward primer containing a fluorescent tag (FAM) was used in a PCR reaction with a wild-type sequence reverse primer. Only mutated DNA will be amplified (203bp), if present, and the PCR reaction product was analyzed using capillary electrophoresis in an automated genetic analyzer. We detected JAK2V617F in 53 cases observed because of erythrocytosis; 29 cases were diagnosed as having a PV, while the remaining cases were identified as secondary erythrocytosis (SE). Notably, most PV patients also have varying degrees of leukocytosis and thrombocytosis. A statistically significant higher Ht level and a WBC and platelet counts were observed among cases with JAK2V617F as compared wild type JAK2 expression (Figure 1).

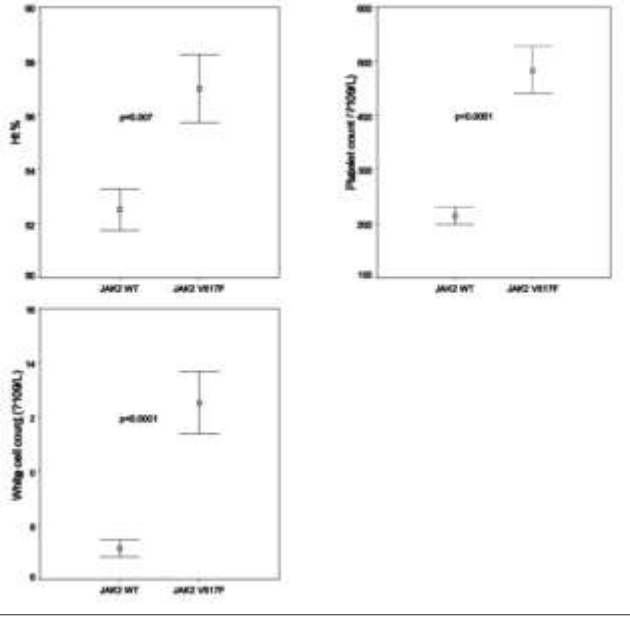

Figure 1.

For calculating the best cut-off values predicting JAK2V617F status, constructing receiver operating characteristic (ROC) curve analysis were performed. We determined 350×10^9/L (AUC=0.916, p<0.0001) and 10×10^9/L (AUC=0.872, p<0.0001) as the best cut-off values for PLT and WBC, respectively. Moreover, 58% was identified as the best cut-off for Ht. On the basis of these results, cases were clustered accordingly. In particular, WBC > 10×10^9/L, PLT > 350×10^9/L and Ht > 50%, predicted 93%, 94% and 70% of JAK2V617F cases (Figure 2). Thus, we designed a risk score based on these results, giving a score 1 to each *abnormal* variable. The devised score system allowed us to split cases in 4 groups with a different incidence of JAK2V617F cases. Since cases scored 3 and 4 had overlapped results, 3 final risk groups were generated (Figure 2).

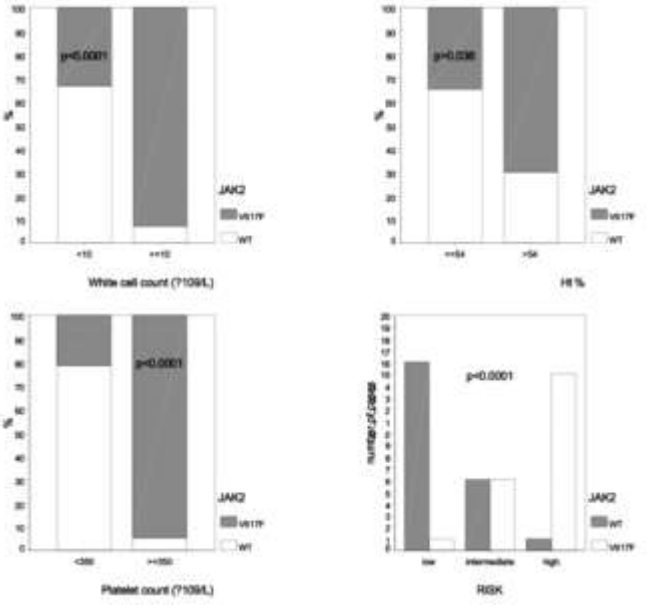

Figure 2.

Low risk and high risk groups showed respectively 1/17 and 15/16 cases with the specific JAK2 mutation, while JAK2V617F equally distributed among intermediated risk group. In 19 cases we performed red cell mass. All 6 cases belonging to low risk group resulted normal to red cell mass determination and all of them were also JAK2 wild type. Conversely, red cell mass confirmed the diagnosis in 3 out of 4 high risk group cases also showing JAK2V617F. Among the 6 cases of the intermediate risk group, 3 with a JAK2 wild type status were also normal for red cell mass determination, while out of the remaining 3 JAK2V617F cases, 2 had also abnormal red cell mass. In conclusion, JAK2 mutational status is highly predictable clustering cases by 3 hematological parameters. However, since the presence of the mutation indicates clonality, JAK2 genotyping should be included in the diagnostic work-up of erythrocytosis, especially with the establishment of the less time consuming PCR-based methods, which can become routinely available in laboratories offering molecular diagnostic services. Finally, red cell mass measurement still may have a role for correctly diagnosing PV cases, but it could postponed in the diagnostic algorithm for patients presented with erytrhocytosis.

PO-185

PRESENCE OF JAK2 V617F MUTATION AND CLINICAL BEHAVIOUR IN PATIENTS WITH ESSENTIAL THROMBOCYTEMIA: RESULTS FROM A SINGLE CENTER STUDY

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Background. It has been reported that an acquired V617F mutation in JAK2 occurs in approximately 30-50% of patients with essential thrombocytemia (ET). Recent reports suggest that the presence of this mutation is associated with clinical features resembling polycythemia vera as well as an increased risk of thrombosis. AIM: the aim of present study was to verify whether patients suffering from ET and positive for JAK2

V617F mutation (mutation-positive) display a distinct clinical behavior from those without JAK2 V617F mutation (mutation-negative). *Methods.* Genomic DNA from peripheral blood granulocytes of patients with ET attending the outpatient Clinic were tested for the presence of JAK2-V617F mutation by an allele-specific Polymerase Chain Reaction (Baxter EJ *et al.* Lancet 2005;365: 1054-61); their clinical records were reviewed for their diagnostic blood counts, thrombotic histories and bleeding events. Thrombotic complications included major thromboses as well as microvascular disturbances. *Results.* Among the 51 ET patients in who was possible to assess the status of JAK2 V617F mutation, 29 (56,8%) were mutation-positive and 22 (43.1%) were mutation-negative. In mutation-positive ET patients blood counts at diagnosis were higher than in mutation-negative ET ndividuals, with a statistically significant difference for hemoglobin concentration (Hb (g/dL): 14.6(+1,4)vs 13.4(+1.5), p=0.003). With a similar median follow-up of 69 and 60 months, respectively for mutation-positive and mutation-negative patients, no difference was observed in the occurrence of thrombotic or bleedings events between the two groups of patients; furthermore, no difference was observed between the two groups for the need of any form of cytoreduction at any time from the diagnosis. *Conclusions.* Our study suggests that the presence of the JAK2 V617F mutation may identify a subgroup of patients with a more pronounced polycythemic pattern of disease presentation; however, this fact seems to do not influence the clinical behaviour of the disease, as by others suggested.

Table.

	All patients	*JAK2 positive*	*JAK2 negative*	*p-value*
Number (*)	51	29 (56,9)	22 (43,1)	
Female/males	28/23	20/9	8/14	NS
Age at diagnosis (years)	58	59	55	NS
Disease duration* (months)	66	69	60	NS
Haemoglobin (g/dL) (±SD)	14.1 (±1,5)	14.6 (±1,4)	13.4 (±1,5)	p<0,01
WBC (×10^9/L) (+SD)	11.1 (±6,7)	12.1 (±8,1)	9,9 (±4,2)	NS
Platelets (×10^9/L) (+SD)	869 (±218)	854 (±197)	890 (±246)	NS
Subjects with thrombosis*	16(35%)	10 (34%)	6 (35%)	NS
Subjects with bleeding* (%)	9 (18%)	6 (21%)	3 (18%)	NS
Subjects with microvascular events* (%)	11 (24%)	9 (31%)	2 (12%)	NS
Patients receiving cytoreduction* (%)	42 (91%)	27 (93%)	15 (88%)	NS

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USEFULNESS OF JAK2V617F MUTATION IN DISTINGUISHING IDIOPATHIC ERYTHROCYTOSIS FROM POLYCYTHEMIA VERA

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Idiopathic erythrocytosis (IE) is characterized by increased red blood cell mass, in the absence of apparent cause of secondary erythrocytosis and sufficient criteria for the diagnosis of polycythemia vera (PV). In order to verify the relationship between IE and PV, we screened IE for JAK2V617F mutation by multiple approaches. Patients were derived from a consecutive series of primary erythrocytosis. Erythrocytosis was defined as an abnormal haematocrit (>51% in men; > 48% in women) with a RBC measurement >25% than the mean predicted value. PV was diagnosed according to the WHO classification. IE was considered as a primary erithrocytosis in the absence of sufficient WHO criteria for PV. DNA and RNA were extracted from fresh or cryopreserved bone marrov (BM) and peripheral blood (PB) cells collected at diagnosis. For each patient, both BM and PB were simultaneously investigated for JAK2V617F mutation by cDNA sequencing and mutation specific PCR. Twenty-six patients were identified as primary erythrocytosis. Of these, 15 fulfilled WHO criteria for PV. The remaining 11 patients were classified as IE. All 11 IE had an elevated haematocrit (median 52%, range 52-58%) with erythrocytosis confirmed by RBC mass measurement. None of the 11 IE displayed any apparent cause of secondary erythrocytosis based on supine arterial oxygen saturation, carboxyhaemoglobin, p50, EPO levels and familial and/or personal antecedent blood counts. None of the 11 IE carried any WHO criteria required for PV diagnosis. All IE cases had normal leukocyte (median 6.4×10^9/L, range 5.8-10.8×10^9/L), platelet (median 197×10^9/L, range 147-320×10^9/L) and serum EPO levels (median 8.5 mUI/mL, range 4.1-13 mUI/mL). Splenomegaly was absent in all IE cases clinically and by ultrasound scanning. Karyotype was normal in 10 IE cases, and indeterminate in one. All IE patients were devoid of BCR/ABL rearrangement and none scored positive for EEC formation. The BM of the only female patient showed a XCI pattern consistent with clonal haemopoiesis by HUMARA assay. In 7 IE patients, BM histology displayed an increased cellularity with prominent erythroid proliferation. In 4 IE cases, BM histology showed panmyelosis with prominent erythroid and megakaryocytic proliferation. IE patients were younger than PV patients (median age at diagnosis 61 ys, range 44-68 ys; versus 67 ys, range 49-89 ys) and predominantly of male sex (male:female ratio 10:1 versus 9:6). No thrombosis at diagnosis was observed in IE. All IE were treated with phlebotomy + aspirin. Median haematocrit during follow-up was 45%. After a median follow-up of 24 months (range 3-46), no IE developed thrombosis or clues of progression to overt CMPD. Thrombosis at diagnosis occurred in 3/15 (20.0%) PV patients. Cases of PV were treated with phlebotomy or cytostatic agents + aspirin. Median haematocrit during follow-up was 44%. After a median follow-up of 33 months (range 3-139), 2/15 (13.3%) PV developed thrombosis and 1/15 (6.6%) PV evolved to myelofibrosis with myeloid metaplasia. Cases of IE were analysed for JAK2V617F mutation. As a first step, JAK2V617F mutation was screened on PB by cDNA sequencing. All 11 IE were negative for JAK2V617F mutation when tested by cDNA sequencing and mutation specific PCR on both PB and BM. For comparison, 15 PV cases were screened for JAK2V617F mutation. Eleven out of 15 (73.3%) PV carried JAK2V617F mutation (6 heterozygous and 5 homozygous). Concordance was 100% when comparing results of cDNA sequencing and mutation specific PCR on both PB and BM samples. Our data demonstrate that JAK2V617F may be a useful molecular marker to distinguish IE from PV among patients presenting with primary erythrocytosis. Absence of JAK2V617F mutation in IE provides a molecular correlate for the clinical distinction between IE and PV.

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CIRCULATING CD 34+ CELLS IN PHILAPDELPHIA NEGATIVE MYELOPROLIFERATIVE DISEASES: A STEADY STATE PARAMETER OR A SPY OF PROGRESSION?

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A debate on the meaning of circulating CD34 positive (cCD34$^+$) cells in myeloproliferative disorders (MPD) is still open. Several studies have suggested a possible diagnostic and/or prognostic role for cCD34$^+$ cells count in patients affected by Myelofibrosis (MF)[1-4] Polycythemia Vera (PV) and Essential Thrombocythemia (ET) (5). We evaluated cCD34$^+$ cells in 124 MPD patients: 30 MF, 29 PV, and 65 ET patients; the diagnosis was made according the PVSG/WHO criteria. cCD34$^+$ cells were also determined in 13 patient with idiopathic erythrocytosis (IE) and in 9 with secondary thrombocytosis (ST). 33% of the patients were de novo diagnoses. The aim of the study was to look for a correlation between cCD34$^+$ cells and MPD progression. cCD34$^+$ cells were quantified using a combination of two monoclonal antibodies: fluorescein isothiocyanate-conjugated anti-CD45 and phycoerythrin- conjugated anti-CD34 (Becton Dickinson, Boston, MA-USA); c-kit expression was also measured. Fifty microliters of blood were incubated with the antibodies for 20 min in tubes holding a known number of microbeads. Cells were determined using FACSCalibur (Becton Dickinson, Boston, MA-USA) by scattering and fluorescence methods, and analyzed shadowing the sequential gating strategy suggested by the International Society for Hematotherapy and Graft Engineering (ISHAGE). We found a mean of 77.8 (range 0-889) cCD34$^+$ cells/microL in MF, 11.9 (0-144.1) in PV, 9 (0-82.3) in ET, 2.8 in IE and 1.7 in ST. According to other reports (2,4), we considered: cCD34$^+$ > 5 cells/microL as an *over upper limit* (OL), < 15 as *discriminative* (DIS) and > 15 as *progression* (PRO) values respectively. In MF (n 30): 5 (17%) were OL, 11 (37%) DIS and 2 (6%) PRO; in PV (n 29): 10 (34%) were OL and 3 (10%) DIS; in ET (n 65): 18 (28%) were OL and 7 (11%) DIS. IE and ST patients had in all cases cCD34$^+$ cells < 5 cells/microL. In all patients cCD34$^+$ cells were monitored at least twice. In two MF with cCD34$^+$ cells > 300 /microL (mean of 819 and 571 respectively) no progression towards leukaemic transformation over a three year observational period occurred. This data suggest that[5]

cells microL can be a discriminative range to distinguishing primary from secondary bone marrow proliferation but 15/microlitro does not be used for differential diagnosis in primary myeloproliferative disorders. Univariate analysis of cCD34+ cells stratified for (a) bone marrow fibrosis (according to Thiele *et al.,* 2005), (b) spleen volume, (c) serum LDH, (d) overall survival and (e) leucocytosis, showed no significant correlation [r=0.3 in (a), 0.3 in (b), 0.6 in (c), 0.1 in (d), 0.4 in (e) respectively]. No correlation was found for patients with mutated JAK-2 as well. In all groups, therapy versus no treatment seems did not affect cCD34+ cells value (r=0.1). Our and other published data support the diagnostic relevance of measuring cCD34+ cells in MPD, and suggest do not consider increased cCD34+ cells as a spy of progression.

References:

1. Barosi G. et al. Blood 2001; 98: 3249-55.
2. Passamonti F. et al. Haematologica 2003; 88: 1123-9.
3. Sagaster V. et al. Haematologica 2003; 88: 1204-11.
4. Arora B. et al. Br J Haematol 2004; 128: 42-8.
5. Andréasson B. et al. Eur J Haematol 2002; 68:189-93.

PO-188

ANTI-IL-5 RECOMBINANT HUMANIZED MONOCLONAL ANTIBODY (MEPOLIZUMAB) FOR THE TREATMENT OF IDIOPATHIC HYPEREOSINOPHILIC SYNDROME (HES).

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Idiopathic hyper-eosinophilic syndrome (HES) is characterized by persistent peripheral blood eosinophilia over 1,500 cells/μL lasting for more than 6 months, in the absence of other apparent aetiologies for eosinophilia with signs and symptoms of organ involvement. Treatment of HES includes corticosteroids, chemotherapeutic agents, interferon-α (IFN-α), and, more recently, imatinib mesylate for the myeloproliferative forms with the involvement of PDGFRA. IL-5 is a cytokine critically involved in regulating several aspects of eosinophils including their production, activation, and tissue recruitment. Mepolizumab is a recombinant humanized monoclonal antibody against IL-5, tested in early clinical trials on patients with asthma and atopic dermatitis. Its role in HES has to be established. A phase I/II study of Mepolizumab in HES proved to be safe and to lower peripheral blood eosinophilia in patients already taking concurrent glucocorticoids. We are presenting our experience with this new and promising drug. We treated three patients enrolled in a phase II clinical trial and one patient out of protocol, with compassionate use. All the patients were tested in respect of the presence of FIP1L1-PDGFRα fusion transcript and they resulted negative after nested-PCR. They were previously treated with imatinib mesylate for a period ranging between two weeks and ten months. None had a stable response. All patients signed an informed consent and were treated with intravenous doses (750 mg) at 4-week intervals. Patient 1 was a 19-year old woman who on diagnosis showed generalized muscle rigidity and pain even with eosinophils count of 20.000/mmc. Her treatment had been primarily oral methyl-prednisolone that resolved symptoms temporarily. Even though she had no relapse of muscle symptoms, she maintained high peripheral blood eosinophilia. Therefore she tested imatinib mesylate, however, without a positive effect and she was subsequently treated with two doses of Mepolizumab. She was resistant also to this therapy. Patient 2 was a 63-year old man with a two year history of idiopathic HES primarily involving biopsy-proven eosinophilic pneumonia and with recurrent asthma symptoms. His treatement with prednisone and imatinib made him stable for ten months. After he relapsed with the same symptoms. During the nine months of Mepolizumab therapy his eosinophils were already < 200/mmc, and he had an increase six months after from the last dose. Patient 3 was a 65 year old man with a long history of idiopathic HES with instrumental pulmonary involvement stabilized with prednisone. He also suffered iatrogen insulin-dependent hyperglicemia. When he started Mepolizumab administration prednisone was reduced and progressively suspended. Until now he maintained stable eosinophil count (< 600/mmc) with a 3-months administration. Patient 4 was a 25-year old woman with sinusitis/nasal polyposis and respiratory difficulty. She had been treated with corticosteroid but she had frequently recurrence of her symptoms also in absence of peripheral eosinophilia. She had a prompt resolution of HES symptoms just after Mepolizumab infusion. No one had adverse event during and after the infusion. Anti-IL-5 antibody appears to be safe in 4 patient with HES. Peripheral blood eosinophil counts is decreased by this therapy, and all the symptoms disappeared after the therapy in all but one patients. Further studies are necessary to establish the exact role and device of Mepolizumab in HES, anyhow it seems to be a good alternative to the corticosteroid therapy in order to prevent the organ damage due to persistent eosinophilia.

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IN VITRO CULTURES SUPPORT HISTOLOGICAL PARAMETERS IN THE IDENTIFICATION OF DIFFERENT SUBSETS OF MYELOFIBROSIS WITH MYELOID METAPLASIA (MMM)

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Myelofibrosis with Myeloid Metaplasia (MMM) is a rare myeloproliferative chronic disorder (CMPD) characterized by clonal hematopoietic proliferation and associated with ineffective and extramedullary hematopoiesis, progressive splenomegaly, bone marrow fibrosis and neoangiogenesis. MMM is also characterized by an increased number of circulating CD34+ cells and the prominent amplification of dystrophic megakariocytes. The molecular basis of the disease has remained elusive although substantial progress has been made regarding the pathogenesis of the associated bone marrow stromal reaction. Twenty patients, previously diagnosed as chronic idiopathic myelofibrosis (CIMF), were re-examined according to the WHO morphological parameters (Vardiman *et al.*, 2001) and classified as prefibrotic-CIMF (p-CIMF) (7 pts), fibrotic-CIMF (f-CIMF) (3 pts), polycythemia vera (PV) (3 pts) and post-polycythaemic myelofibrosis (PPMF) (7 pts). The median age was 67 yrs (range: 45-84), generally presenting cytogenetic abnormalities and treated with chemotherapy. CD34+ cells were quantified in peripheral blood (PB) according to ISHAGE gating strategy. Clonogenic precursors were documented both on bone marrow mononuclear cells (BM) and on cells extracted from the bone marrow biopsy (BMB) using either mechanical (flushing) and enzymatic (trypsin) approaches. Fibroblast progenitor cell number (CFU-F) was studied seeding 1×10^6 cells in IMDM medium supplemented with 20% fetal bovine serum. Myeloid progenitor cell number (CFU-GM) was assessed in semisolid methylcellulose medium seeding 5×10^5 cells/ml. Microvessel density hot spots (MVD) was evaluated according to Weidner *et al.*, 1991. Peripheral blood CD34+ cells were higher in all classes in comparison with normal subjects but a significant decrease in the median values was observed from p-CIMF (110 CD34+ cells/μl) to f-CIMF (42 CD34+ cells/μL) and from PV (393 CD34+ cells/μl) to PPMF (178 CD34+ cells/μL). The committed myeloid progenitor cells (CFU-GM) showed a similar trend in both in bone marrow aspirated (BM) and bone (BMB): the median values of CFU-GM were respectively 75.5 in p-CIMF, 59 in f-CIMF, 41.5 in PV and 19 in PPMF for BM and 12 in p-CIMF, 111.5 in PV and 10 in PPMF for BMB. This trend suggested that f-CIMF and PPMF could represent late stages of the disease in which hematopoietic cells have been progressively replaced with fibroblasts. The median number of fibroblast precursors (CFU-F) was much lower in BM (8) than in BMB (112) in all groups of patients even though these findings may be related to sampling artefacts due to the difficulties of bone marrow aspiration in MMM patients. However, in both BM and BMB, median number of CFU-F increased from p-CIMF (1 in BM and 67.5 in BMB) to f-CIMF (18 in BM and 176 in BMB), suggesting an active progression of myelofibrosis; on the contrast, BMB CFU-F significantly decrease from PV (167.5) to PPMF (36), indicating a progressive exhaustion of the fibroblast precursors. Moreover, MVD performed in a larger group of patients (n=50) showed higher median values than normal controls particularly evident in PPMF (33.8 versus 6.6), thus pointing out an inverse correlation between fibrosis and angiogenesis. In conclusion, our *in vitro* data support the WHO classification and suggest that f-CIMF and PPMF could be the fibrotic exit of two distinct MPDs, respectively p-CIMF and PV. Therefore, as the ethiopatogenesis of the two diseases seems to be intrinsically different, our results could be useful diagnostic tool and should have clinical application.

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MUTATIONAL ANALISYS OF JAK2 VAL617PHE IN A NEW SERIES OF CHRONIC MYELOMONOCYTIC LEUKEMIA AND RELATED ATYPICAL MYELOPROLIFERATIVE DISORDERS

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Background. A recurrent somatic activating mutation in the non-receptor tyrosine kinase JAK2 V617F has been described in chronic myeloproliferative disorders (CMPD) i.e. Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Myelofibrosis with Myeloid Metaplasia (MMM). Incidence of JAK2 V617F mutation in the myelodysplastic/myeloproliferative (MDS/MPD) category, or in MDS has not been definitely established. In MDS, JAK2 V617F mutations seem to be strictly associated with refractory anemia with ringed sideroblast and thrombocytosis (RARS-T). *Aim.* To investigate the prevalence of JAK2 V617F mutation in CMML/aCMML/related atypical MPDs at diagnosis using a dHPLC technology. *Materials and Methods.* Samples were obtained from peripheral blood or bone marrow of 13 patients (10 Males, 3 Females, mean WBC count 18×10^9/L, mean Platelets count 313×10^9/L. Splenomegaly was detected in two patients. In two other patients mutational analysis revealed KRAS2 G34C (Gly12Arg) and NRAS C181A (Glut61Lys). Karyotype was normal in 8/9 evaluable patients. In the remaining case FISH detected trisomy of chromosome 8. Genomic DNA from patients, and from a positive sequenced control obtained by a PV patient with V617F, was extracted from blood using standard procedures. PCR fragments of JAK2 exon 12 were amplified using the following oligonucleotide primers: JAK2-F (5' TTCCTGTACCACTCTTGCTC 3'), JAK2-R (5' TTTCAGGATCACAGCTAGGT 3'). The 12th coding exon with flanking intron sequences of the JAK2 gene were screened for Val(617)Phe mutation by dHPLC (Wave™ System, MD Transgenomic Inc., Omaha, Nebraska, USA). Samples were kept cool until 10 μL were automatically inserted into a preheated reversed phase column based on non-porous (polystyrene-divinylbenzene) particles (DNA-Sep, Transgenomic, San Jose, USA). Gradient elution and melting temperature conditions were determined using WaveMaker Navigator™ version 1.5.4 software (Transgenomic). On the basis of the PCR product (419bp) nucleotide sequence analysis program calculate the melting temperature of the domains contained in the sequences of interest at 54.2 °C. Electropherograms from patients were compared with normal and positive sequenced controls. *Results and Conclusions.* Mutational analysis did not detect JAK2 V617F in our series confirming precedent data on the rare occurrence of this genomic lesion in CMML/aCMML/related atypical MPDs. Cytogenetic, Molecular and Clinical Hematological findings reported in MDS/MPD and CMML with JAK2 V617F mutation.

Table.

N. Patients /N.Positive	*%Positive Cases*	*Concomitant NRAS-KRAS*	*KARYOTYPE in JAK2+*	*Splenomegaly*	*Cytopenia*
119/3	3%	n.a.	n.a.	n.a.	-
47/1	2%	1/1	n.a.	n.a.	-
97/17	17.1%	n.a.	n.a.	n.a.	n.a.
78/8	7%	2/8	(7) Normal; (1) +8	?	-
4/3	75%	n.a.	n.a.	?	-
104/5	4.8%	n.a.	(1) t(8;9)(q22;p24)	?	-
3/3	100%	n.a.	(1) del13q; (1) normal	1/3	-

This and previous reports were not able to demonstrate any specific correlation between JAK2 and cytogenetic, molecular or clinical findings in CMML and related malignancies. Karyotype (trisomy 8), molecular data (RAS mutations), and clinical-hematological features (splenomegaly, and absence of cytopenia) which have been found in JAK2+ cases (see Table) were also present in our negative series, suggesting that these findings cannot be used as indicators of JAK2 status in CMML or other related atypical myeloproliferations. This study confirms dHPLC as a sensitive hight-throughput method to investigate V617F in genomic samples. New candidate genes need to be studied in order to better delineate the genetic profile of this MDS/MPD subgroup.

Fondazione Cassa Di Risparmio di Perugia; PRIN_MIUR (Ministero Istruzione, Università e Ricerca Sientifica); Associazioni Sergio Luciani, Fabriano.

References

Steensma et al, Blood 2005.
Johan et al, British Journal of Haematology, 2005.
Jones et al, Blood 2005.
Levine et al, Blood 2005.
Kremer M et al, Leukemia 2006.
Szpurka et al, Abstract 396 2005.
Remacha et al, Haematologica 2006.

PO-191

IMATINIB-MESYLATE THERAPY FOR SISTEMIC MASTOCYTOSIS: RELATIONSHIP TO C-KIT MUTATIONAL STATUS

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Background. Systemic mastocytosis(SM) includes an heterogeneous group of neoplastic disorders characterized by abnormal mast cell accumulation in various tissues and by both indolent or aggressive clinical outcome. SM has been supposed to be associated with two classes of constitutive activating c-kit somatic mutations: the so-called *enzymatic site* type (EST)mutations, affecting the structure of the catalytic portion of the kinase (e.g. D816V), and the *regulatory* type (RT) mutations, affecting the regulation of an otherwise normal catalytic site (e.g. V560G). *Aim.* Since c-kit is a transmembrane receptor-type tyrosin kinase, we aimed to test the hypothesis of whether an inhibitor blocking constitutive c-kit activation, such as imatinib, could have therapeutic activity in SM and whether c-kit mutational status could have importance for response. *Methods.* We report on nine patients treated with imatinib who met the major classification criteria for SM, who were symptomatic and who had a biopsy-proven evidence of disease. Six of them were male and three female, age ranged from 33 to 76. Organ involvement included skin, bone, stomach, bowel, bone marrow, spleen, lymphnodes, lung and heart, variously combined in different patients. Two patients had elevated eosinophils in their peripheral blood (45% and 19%, respectively). All patients resulted to be negative for the FIP1L1-PDGFRα fusion transcript, which characterizes hyperosinophilic syndromes (HES) with high sensitiveness to imatinib. The drug was given at the dose of 400 mg/die for a median period of 3 months (range 1.5-5 months). *Results*. Before therapy, mastocyte cells from six patients were found positive for D816V mutant of c-kit. None of these patients achieved significant clinical benefits from imatinib therapy. Regarding the three subjects without D816V c-kit mutation, one patient, with elevated count of eosinophils in peripheral blood, showed an initial response to imatinib, but lost it after one month from the beginning of treatment. A second patient had a significant reduction of splenomegaly, but rapidly relapsed and died of progressive disease. The third patient without D816V mutation showed a prolonged significant reduction of skin lesions and marrow mastocytosis. *Conclusion.* Our observations suggest that imatinib treatment is ineffective for SM characterized by EST c-kit mutation, even if associated with hypereosinophilia, while patients without such a mutation may have some clinical benefit. A complete evaluation of the molecular pattern in SM treated with imatinib, including RT mutations (e.g. V560G and F522C), is currently in progress.

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JAK2 V617F ACTIVATING TYROSINE KINASE MUTATION IN MYELOPROLIFERATIVE DISORDERS

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A missense somatic mutation in JAK2 tyrosine kinase gene (V617F) has recently been reported in myeloproliferative disorders (MDPs) including polycythemia vera (PV), essential thrombocythemia (TE) and idiophathic myelofibrosis (IMF) strongly suggesting its pathogenetic role in these diseases. JAK2 V617F results in costitutive activation of the tyrosine kinase, phosphorylation of STAT5 and factor-independent growth of hematopoietic cells. The mutated allele was not observed in blood from healty individuals and in buccal cells from MDP patients, demostrating that the mutation in MDPs is acquired in a hematopoietic progenitor. We screened 50 MDPs (18 PV, 12 TE, 8 IMF, 5 secondary thrombocythemia, 2 polyglobulia, 5 myelodysplastic syndrome). The exon 12 of JAK2 gene was amplified from genomic DNA and amplification products were directly sequenced by *cycle sequencing* approach (BigDye Terminator Applied Biosystems). Sequences of primers utilized for the amplification and sequence reactions were designed according to the literature. We detected JAK2 V617F in blood DNA from 11 patients (22%). The proportion of mutated cases per disease subtype was 10 (55,5%) of 18 PV and 1 (12,5%) of 8 IMF; no mutated cases were observed in our small series of TE. We detected 5 (45,4%) homozygous mutants and all these cases showed LOH at 9p24 (D9S288). Our results on mutated status of JAK2 are in agreement with the published data. Although the role of JAK2 V617F in the pathogenesis of myeloproliferative disorders is clearly documented, some open questions remains. The central issue to be address is if the mutation plays a role of primary cause of myeloproliferative disorders or is a secondary event associated with disease progression. The hierarchical location of the mutation is important in view of development of specific inhibitor for V617F to be used in signal transduction therapy.

Stem Cell Transplantation I

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CHILDHOOD ESSENTIAL THROMBOCYTHEMIA EVOLVING IN IDIOPATHIC MYELOFIBROSIS

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Myeloproliferative syndromes (MPS) are clonal stem cell disorders resulting in excessive proliferation of one or more cell lineages; they occur in children much less commonly than adults. According to the predominant proliferating cell lineage the primary MPS are classified into chronic myelogenous leukemia, polycythemia vera, familial erythrocytosis, essential thrombocythemia, familial thrombocytosis, juvenile myelomonocytic leukemia, idiopathic myelofibrosis, transient myeloproliferative disorder of Down syndrome, idiopathic hypereosinophilic syndrome, mastocytosis. The essential thrombocythemia (ET) in particular is extremely rare in children. Thrombosis is the most common complication in adults but a low number of children develop thrombohemorrhagic phenomena. A girl was referred for a high platelet count detected on routine blood testing since he was 3 years old. The mean platelet count was $600\times10^3/\mu L$ but she was completely asymptomatic. The bone marrow aspirate was hypercellular, with numerous clusters of megakaryocytes and platelet clumps; colony study showed that spontaneous BFU-E were present; the chromosomal analysis was normal and bcr-abl rearrangements were negative. A diagnosis of childhood essential thrombocythemia was made. We excluded familial thrombocythemia, which is common among children with primary thrombocytosis and appears to be a different disease from ET and with a more benign course. A thrombophilic study didn't show hyperhomocysteinemia, Protein S and C deficiency, activated protein C resistance, factor V Leiden G1691A and prothrombin Gene G20210A mutations; instead the girl was heterozygous for methylenetetrahydrofolate-reductase C677T mutation. An abdominal ultrasound at the age of nine, fifteen and twenty years old showed slight splenomegaly. During the follow up the platelet count remained only moderately elevated. A watch-and-wait strategy seemed appropriate in this asymptomatic case and low-dose aspirin has been used. Three months ago, at the age of twenty-three, the young patient referred an abdominal pain and mild oral bleeding; a full blood count showed hemoglobin 12.9 g/dL, mean corpuscular volume 82.4 fl, leucocytes 18.6×10^3/microL, neutrophils 13.1×10^3/microL, lymphocytes 2.7×10^3/microL, platelets 1025×10^3/microL. An abdominal ultrasound showed increased splenomegaly (maximum diameter 16 centimeter and surface 80 cm^2). The peripheral blood demonstrated giant platelets, often aggregated, and hyperlobulated granulocytes. The bone marrow examination showed increased marrow megakaryocytes, increased eosinophil and basophil precursors and the trephine biopsy cluster of megakaryocytes with hyperlobulated forms and above all an initial myelofibrosis, so that the histopathologist leaned more towards an initial idiopathic myelofibrosis (IMF) than for a ET. Spontaneous BFU-E were present, the chromosomal analysis was normal and bcr-abl rearrangements were negative. Leukocytic alkaline phosphatase was abnormal high (score 215) and the positivity for V617F JAK2 mutation, detected by PCR-direct sequencing using DNA from the granulocyte lineage, was demonstrated. We didn't find leukoerythroblastic blood reaction and tear-drop erythrocytes on blood count, typical of IMF and the absolute content of $CD34^+$ cells in the peripheral blood wasn't high (7.3×10^6/L). Since a continuos platelet count over 1000×10^3/microL, about $1400\text{-}1600\times10^3/\mu L$, even if the patient was always asymptomatic, after a monitoring of heart function, she recently started therapy with anagrelide. This drug seems to be a promising approach for children and young adults with ET/IMF. It should be considered as a first-line therapy because of the selective activity against platelet production, mild toxicity, absent cancerogenic and leukemogenic effect and the good compliance by patients due to the oral administration. At the moment we have activated a research for hematopoietic stem cell transplantation (HSCT), the only available curative option for ET/IMF in this so young patient, since the girl hasn't familiar potential donor.

PO-194

ALLOGENEIC STEM CELL TRANSPLANTION IN MULTIPLE MYELOMA (MM)

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Background. HD chemo/radiotherapy followed by autologous stem cell transplantation (SCT) has been associated with improved outcome in MM. Unfortunately, following autologous SCT almost all pts had progressive disease. *Aims.* We evaluated the feasibility, efficacy and toxicity of allogeneic stem cell transplantation for 26 (15 M, 11 F) newly diagnosed stage IIIA(n=22)-IIIB(n=4) multiple myeloma patients (pts) up to the age of 65. Twenty-one and 5 pts underwent a matched sibling donor allogeneic transplant after a nonmyeloablative or myeloablative regimens, respectively. Twenty pts were treated with autografting followed by reduced intensity conditioning allotransplantation. All these patients received HD Melphalan (200 mg/mq) followed by autologous PB-SCT. After a median of 90 days, the pts underwent RICT (Fludarabine + 2 Gy TBI). Acute GVhD prophylaxis consisted of MM and cyclosporine. Chimerism analysis was performed using STR-PCR and donor engraftment was evaluated at day +15,+30,+45,+60,+90 on unfractionated BM cells. All pts received a HLA identical donor mobilized PBSC and the graft contained a median of 3,3 x 10E6 (range 1-6,8) CD34+ cells/kg body weight. After RICT, on day +15, 3 (13%) pts showed a complete donor chimerism; on day +90, 21 (90%) showed a complete donor chimerism; two pts with mixed chimerism received a DLI on day +30 and one of these achieved full donor chimerism. Five pts received a conventional hematopoietic SCT, the pre-transplant high-dose preparative regimen included CY+TBI. On day 0 all collected PBSCs were infused. GVHD prophylaxis included cyclosporine and short-course methotrexate; All patients showed a complete donor chimerism at the time of engraftment. *Results.* For RICT pts grade I-II and III acute GVHD occurred in 5 (24%) and 1 (4%) pts respectively. Five patients (24%) developed a mild and 4 (19%) an extensive chronic GVHD. After RICT 8 pts (38%) achieved CR and they are in CCR at +60,+60,+54,+52,+,49,+23,+17 and +18 months; 2 (9%) pts show near CR and 5 (24%) are in PR. Six (28%) pts not in CR showed a progressive disease and 3 (14%) of these died. With a median follow-up of 23 months, 18 (86%) are alive. Instead the pts that received a myeloablative conditioning regimen, grade II-IV acute GVHD occurred in 2 patient. All pts developed mild chronic GVHD. One patient relapsed and died 24 months after allogeneic SCT. To date, 4 patients are alive and 3 of them are in CCR at + 31 and +7 months; one patient are in PR and one showed a progression of disease. *Conclusions.* We demonstrated that survival after allogeneic transplantation is favourable: 73% of all pts achieved CR or PR. Neither a patient died after allogeneic SCT, considering the old age of the patients that received RICT. Pancytopenia after RICT was minimal and sustained allogeneic stem cell engraftment occurred in 95% of patients. A good correlation between GVHD, full chimerism and remission was found. All patients in CR or NCR developed acute/chronic GVHD and the presence of GVHD correlated with a lower relapse rate. In all patients the achievement of CR was gradual and a constant regression of the monoclonal band was observed.

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MOBILIZATION OF ENDOTHELIAL PROGENITOR CELLS IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES AFTER TREATMENT WITH GRANULOCYTE COLONY STIMULATING FACTOR AND CHEMOTHERAPY FOR AUTOLOGOUS TRANSPLANTATION

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Accumulating evidences show that the peripheral blood (PB) of adults contains a population of circulating bone marrow-derived endothelial progenitor cells (EPCs) which can migrate from the bone marrow, proliferate and differentiate into mature endothelial cells (ECs). In PB, EPCs are rare events representing between 0.01% and 0.0001% and can be identified by the co-expression of CD34, vascular endothelial growth factor receptor 2 (VEGFR2), and AC133 (also known as CD133). The increasing interest for EPCs is mainly due to their possible use in cardiovascular diseases. To this extent it has been shown that in patients with acute myocardial infarction the intra coronaric injection of EPCs can ameliorate post ischemia remodeling, while in patients with chronic hind limb ischemia after autologous stem cell transplantation they improve pain and of cutaneous ulcers due to hypoxia. It has been shown that Granulocyte Colony Stimulating Factor (G-CSF) or Granulocyte-monocyte Colony Stimulating Factor (GM-CSF) can mobilize EPCs into the PB. However there is insufficient data concerning the mobilization and collection of EPCs during $CD34^+$ cell mobilization. In this study we have evaluated EPC mobilization and collection in subjects with hematological diseases, treated with GCSF in combination with chemotherapy for CD34+ cell mobilization. The study population comprised 47 patients in clinical complete remission according to CT scan and/or bone marrow biopsy [31 cases of Non Hodgkin Lymphoma (NHL) and 16 cases of Multiple Myeloma (MM)] and all patients were treated with Cyclophosphamide (4 grams/meter square on day +1) and GCSF (5 micrograms/kilogram/die from day +4 until day +13). PB CD34+/CD45+ cells were enumerated by flow cytometry according to standard techniques on day –1, +1, +7 and from day +9 until day +13 and on day + 20. PB EPC were enumerated on day –1 and from day +9 as endothelial cultures (CFU-endo) and by flow cytometry as CD34+ cells co-expressing AC133 and VEGFR-2. In this study, 80% of patients had a successful mobilization of CD34+ cells. MM patients were older than NHL patients (60.12 years ±6.96 SD in MM vs. 45.19 years ± 10.55 SD in NHL $p<0.01$). No difference was observed concerning the other principal clinical-biological characteristics. The number of PB EPC as assessed as CD34+/VEGFR2+/CD133+ cells showed a peak on day +10. This peak paralleled that of PB CD34+/CD45+ cells. A strict direct correlation was observed between PB CD34+ cells and CD34+/VEGFR2+/CD133+ cells during all days of mobilization ($r=0.99$ $p<0.0001$).Significant higher levels of CD34+/VEGFR2+/CD133+ cells/kilogram have been collected on days +9 and +10 in comparison to day +11 ($p=0.03$ and $p= 0.04$, respectively). Concerning CD34+/VEGFR2+/CD133+ cells, in NHL patients we showed an earlier mobilization of EPCs in patients with limited disease (stage I and II) than in patients with advanced disease (stages III and IV) ($p=0.035$), while no correlation between stage and mobilization was observed in MM patients. No other significant correlations between CD34+/VEGFR2+/CD133+ cells mobilization and clinical parameters were observed in NHL and MM patients. On the whole an average of 23.7×10^6 CD34+/VEGFR2+CD133+ cells have been collected (range 12.1- 41.76×10^6). The number of cells collected by this procedure is much larger than that currently used in reparative medicine applications which is between 1.5 and 5.2×10^6 CD133+ cells. Concerning CFU-endo, no significant peaks have been observed in MM and NHL patients. No significant correlation was observed between CFU-endo and CD34+ cells or CD34+/VEGFR2+/CD133+ cells. In conclusion, we have shown that in hematological diseases Cyclophosphamide in combination with GCSF allows the mobilization and collection of large numbers of CD34+/VEGFR2+/CD133+ cells along with CD34+ cells. These cells might represent a promising therapeutic tool for future possible reparative medicine applications in these patients.

PO-196

MARKED TELOMERE LOSS IN AUTOGRAFTED PATIENTS COULD PREDICT SECONDARY MYELODISPLASTIC SYMDROMES

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Background. Secondary myelodysplastic syndrome (sMDS) and acute myelogenous leukemia (AML) may occur following autologous stem cell transplantation (ASCT). A retrospective analysis at our Institution showed a 5-year cumulative probability of developing sMDS/AML of 4.8% for lymphoma patients who underwent high-dose chemotherapy (HDS) and ASCT (Bono D. *et al.,* Blood 104:11, 2004). The molecular pathogenesis of sMDS/AML remains uncertain and we still lack a simple and wildly-applicable method which could predict the rising of sMDS/AML. It is well known that accelerated telomere loss may be induced by replicative stress. We recently observed a marked telomere shortening in hematopoietic cells of autografted lymphoma patients that persists at long-term. AIMS. In this study, we assessed post-ASCT telomere dynamics in order to verify whether accelerated telomere loss

of hematopoietic stem cells might underlie the pathophysiology of sMDS/AML. *Patients and methods*. We retrospectively analyzed telomere restriction fragments (TRF) length of bone marrow (BM) cells from 35 long-term survivors autografted lymphoma patients (22 males and 13 females) and 51 healthy donors (31 males and 20 females). Among the 35 autografted patients, 5 developed a secondary hematological malignancy (2 AML, 2 clinical sMDS, 1 moderate but persistent pancitopenia with cytogenetic abnormalities). Patient median age at follow-up was 51 years (range: 24-68 yrs) while healthy donors had a median age of 53 years (range: 18-82 yrs). The 5 sMDS/AML patients do not differ from the other autografted patients in terms of demographical and clinical features. In particular, no differences were found in terms of CD34+ve cells reinfused. Median follow-up from ASCT was 6 years (range 3.5-10 yrs). All patients were in continuous complete remission since autograft at the time of TRF length analysis. TRF length was evaluated by Southern blot. *Results*. TRF length of patients was compared to that of the healthy donors and a significant telomere loss was observed in autografted patients, as expected (Figure, panel A, squares and grey circles). Indeed, median TRF length of autografted patients was 6739 bp (range: 4472-9004 bp) while median TRF length of healthy donors was 7791 (range: 5836-11058 bp) (p<0.005). Of note, all the patients who developed sMDS/AML had a TRF length shorter than the median TRF length of autografted patients (Figure panel A, black circles). Thus, comparing only age-matched autografted patients (12) and healthy subjects (19), we found that sMDS/AML patients had telomeres significantly shorter than both healthy subjects and autografted patients (median TRF length 5738 bp, range 4472-6493 bp, p<0.0005 and p<0.01, respectively) (Figure, panel B). *Conclusions.* i. patients with short telomeres seem to have a higher risk of developing secondary hematological malignancies, suggesting that telomere erosion may be relevant to the pathogenesis of sMDS; ii. if these results will be confirmed on larger and independent populations, they will add further knowledge into the pathogenesis of sMDS.

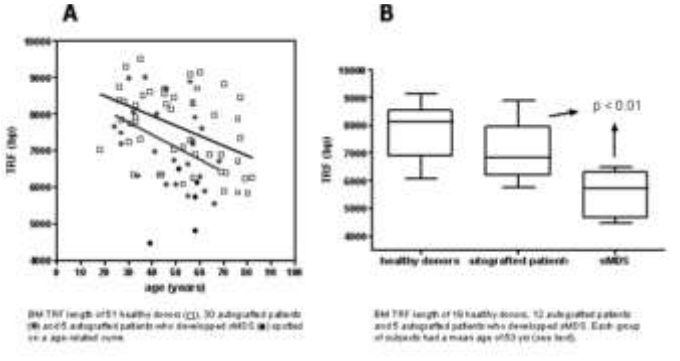

Figure 1.

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PURIFIED T DEPLETED PERIPHERAL BLOOD AND BONE MARROW CD34 TRANSPLANTATION FROM HAPLOIDENTICAL MOTHER TO CHILD WITH THALASSEMIA

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Approximately 60% of thalassemic patients can not apply to *gene therapy today* which the insertion of one allogenic HLA identical stem cell into the empty bone marrow as the vector of the normal gene for β globin chain synthesis. 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 fourteen 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 Sangstat 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.* All patients are alive. Three patients rejected the transplant and are alive with thalassemia. Eleven\ patients are alive disease free with a median follow up of 28 months (range 9-44). None of the seven patients showed AGVHD and CGVHD. 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.

PO-198

DIFFERENT γ-δ T CLONES SUSTAIN GVM AND GVH EFFECTS IN MULTIPLE MYELOMA PATIENTS AFTER NON-MYELOABLATIVE TRANSPLANTATION

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γ/δ T lymphocytes have many features that distinguish them from the α/β T cells: a faster turnover rate, a more rapid expansion after infection, the capacity of regulating CD4$^+$ and CD8$^+$ T-cell function and recognizing antigens directly, without requiring antigen processing or presentation. Different α/β T clones have been reported to be responsible for the control of neoplasia (GVM) and of the graft-versus-host disease (GVHD) in patients who underwent allogeneic transplantation for multiple myeloma. Reduced-intensity conditioning, which relies on graft-versus-myeloma effect, is one of the strategies currently developed in order to achieve a stable engraftment, to further reduce recurrence rates as well as transplant-related-mortality (TRM). Because of the well-known anti-cancer activity of γ/δ T lymphocytes in solid tumors, we planned to follow TCR repertoire, with the aim of correlating the possible emergence of different T clones with the most relevant clinical events (in particular acute and chronic GVHD and minimal residual disease) in a series of 20 patients that underwent autologous/non-myeloablative allogeneic transplantation. The tandem autologous/non-myeloablative transplant program was shown to be feasible for all enrolled patients. The 2-year OS rate from NMT was 58%. The failure of autologous transplantation did negatively affect survival after NMT (p=0.001).

The overall PFS at 24 months from the NMT was 51%. In univariate analysis, a shorter PFS was associated with advanced stage at diagnosis (p=0.03) and with failure of PBSCT (p=0.048). NMT showed an acceptable toxicity: eight patients (40%) developed aGVHD grade 1-4 during the first 42 days of follow-up. Six patients (30%) developed cGVHD at a median of 145 days, with two cases of extensive cGVHD. After a median follow-up of 35 months, seven patients (35%) had died, three of them due to disease progression. Actuarial incidence of 100-day TRM was 20%; it was not significantly influenced by the aGVHD occurrence. After NMT, 11 patients (55%) reached PCR-negativity, with a median time of 6 months. Interestingly, in our series MRD eradication had a favorable impact on clinical outcome, either on OS or on PFS: 76% of patients who achieved molecular remission were still alive 20 months after allogeneic transplant, versus 34% of those that had a persistently detectable IgH rearrangement (p=0.03). Moreover, all five progressed patients (100%) were IgH-positive before clinical progression, versus 33% of cases with stable disease (p=0.02). After the non-myeloablative conditioning regimen adopted, 25% of patients achieved full donor (FD) chimerism before day +100, 42% before day +200 and 56% by 24 months after graft. In our series, level of chimerism did not correlate with the status of disease or aGVHD occurrence. In order to better interpret fluorescent PCR results, we applied the spectratype complexity score (SCS) either to healthy adults or to our transplanted patients. Mean value of the number of peaks detected in the control group plus two standard deviations were adopted as cut-off for defining a TCR spectratype as polyclonal. No case showed a SCS comparable with that measured in healthy controls, thus demonstrating that all patients presented a skewed T-cell pattern during the entire follow-up, until to 18-24 months after the graft. Interestingly, no evident increase in the SCS was detected at evaluated time points. During follow-up, 6 of the 13 patients evaluable for TCR profile developed an aGVHD episode; in 5 of them, this event fitted with a modification of the TCR profile, with the appearance of a new T-cell prominent clone. Interestingly, in the three cases that resolved a GVHD, the new-appeared T-clone disappeared when the aGVHD disappeared. Five patients developed a cGVHD; in only two of them, the cGVHD (in both cases limited), was accompanied by the appearance of a new TCR profile. Changes in TCR rearrangement were also evaluated in relation to the emergence of infective complications, but no new T clones appeared. Analogously, the chimerism status did not correlate with the modification of TCR profile. Regarding MRD, in 7 of the 9 patients that achieved the PCR-negativity, the appearance of a new predominant δ-TCR peak was concomitant to the disappearance of the patient-specific IgH clone. Interestingly, in these cases the emerging peak was identical. Serial bone marrow samples from three patients were tested by immunofluorescence assay for T δ cells infiltration. In all samples harvested before allogeneic transplantation, a very weak reactivity for the anti-δ TCR was detected. Interestingly, when we analyzed the two samples where the emergence of a new molecularly detected T-cell δ clone has been concomitant to the MRD eradication (one at 3 and one at 6 months after graft), the immunoreactivity resulted strongly enhanced in both cases, thus really confirming the presence of a significant number of T δ cells in the bone marrow. No significant modifications of the reactivity were noted in those samples where the emergence of a clone was not detected by fluorescent PCR. These results would support the hypothesis that independent T γ/δ clones may be involved in both GVM and aGVHD.

PO-199

HUMAN SERUM FOR ISOLATION AND EXPANSION OF MESENCHYMAL STEM CELLS FOR CLINICAL USE

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Mesenchymal stem cells (MSCs) are promising candidates for cell-based therapies. One major obstacle for their clinical use is the biosafety and potential immunogenecity of fetal bovine serum (FBS), which is a crucial part of all media currently used for the culture of MSCs. To address this problem we evaluated media containing human serum (autologous and allogeneic), at concentration of 10% and compared it to 10% selected FBS and to MyeloCult, medium supplemented with FBS and horse serum, that we routinarly used for selection and expansion of MSCs. Isolation of MSCs was performed by seeding bone marrow mononuclear cells (MNCs) to generate a stromal layer, after two weeks MSCs were subcultured by treatment with trypsine and seeded into fresh medium at low-density. After passage 3 and until passage 10, cells were counted, studied by flow cytometry and cultured for osteogenic and adipogenic differentiation. Moreover we selected CD271 (LNGFR) positive cells from MNCs of the same bone marrow to isolate MSCs and expanded cells with MyeloCult, 10% human serum, 10% FBS and with the NH (MACS) medium. We replating these cells after semi-confluence until passage 10, then we counted cells, studied cells by flow cytometry and cultured cells for osteogenic and adipogenic differentiation at each passage. Isolation and expansion of MSC from CMNs, after three-six passages, appeared best in MyeloCult medium e in human serum at 10%, respect FBS at 10%. Morphologically, MSC isolated and expanded in human serum and FBS were bigger and richer of macrophages at the first, second passage respect MSCs with MyeloCult, but after four, five passages MSCs expanded in human serum were in general CD45 negative, CD14 negative and CD34 negative and SH2 and SH3 positive and showed similar osteogenic and adipogenic potential to MSCs in MyeloCult. MSCs isolated by CD271 positive selection showed a great proliferative potential in all media, in one experiment the expansion was the best (one log more) in human serum until passage 6. MSCs expanded after passage 1 were CD45 neg, CD14 neg and CD34 neg and SH2 and SH3 positive and retained their immaturity as shown by osteogenic and adipogenic differentiation. In conclusion growth of MSCs is feasible in medium containing human serum without addition of growth factors. CD271 cell positive selection resulted in a cell population with a great proliferative potential that rapidly expanded, also with human serum, and retained their multipotential differentiation.

PO-200

CLINICAL RELEVANCE OF INTRAHEPATIC HBV DNA IN HBSAG-NEGATIVE BCAB-POSITIVE PATIENTS UNDERGOING STEM CELL TRANSPLANTATION FOR HEMATOLOGICAL MALIGNANCIES

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Hepatitis flare-up due to hepatitis B virus (HBV) reactivation is a well-recognized complication associated with chemotherapy. Data on the clinical impact of HBV DNA in liver tissue of HB surface Antigen (sAg)-negative patients undergoing immunosuppression are scanty. We investigated the presence and the clinical significance of intrahepatic HBV DNA in 39 consecutive adult patients (20 males/19 females) who had had HBV contact without signs of active infection, and were scheduled to receive hematopoietic stem cell transplantation (HSCT) for hematological malignancies (acute leukemia= 20, chronic leukemia= 10, lymphoma= 9). After signed informed consent, all patients underwent liver ultrasound-guided fine needle cutting biopsy (modified Menghini needle, diameter 1.2 mm). The viral genome was investigated by a well-standardized highly specific in situ molecular hybridization test; it was expressed as percentage of positive hepatocytes by visual inspection. All patients lived in southern Italy, an area considered endemic for HBV infection. At the time of hematological malignancy diagnosis and liver biopsy, the serological viral status assessment by conventional assays showed all 39 patients as being antibodies against HB core Ag (anti-HBc)-positive, and HBsAg- and HBV-DNA-negative. The biopsies were uneventful and suitable for histology according to Ishak's criteria. Nineteen patients were found to harbor the HBV genome (median of positive hepatocytes: 40%, range 5-90). None had immunoperoxidase staining for HBsAg and HBcAg, nor evidence of chronic hepatitis or cirrhosis. No hybridization signals were detected in the biopsy specimens of the remaining 20 patients. After biopsy, patients received standard conditioning regimens and allogeneic (n=14) or autologous (n=25) HSCT. Then, they were observed for a median of 24 months (range, 9-48) without active prophylaxis against HBV, monitoring liver function and viral markers monthly. During the follow-up, no patient in the in situ hybridization negative group had HBV-related hepatitis, while four patients in the in situ hybridization positive group developed overt hepatitis B (median aminotransaminases 500 IU/L, range 250-2000) and sero-converted to HBsAg- and HBV-DNA-positive status, with median onset of five months (range, 3-11) from allogeneic (n=2) or autologous (n=2) HSCT; in these patients, the infected hepatocytes at the time of biopsy ranged from 50 to 90%. After a median of four months of lamivu-

dine treatment (100 mg daily), transaminase levels normalized in all cases. These four patients received HSCT having occult HBV infection confined to the liver; viral activation likely occurred in the context of post-transplantation immunosuppression. Polymerase chain reaction analysis on DNA extracted from the pre-transplantation paraffin-embedded liver specimens showed intact S, C and X HBV genes in all four patients, indicating the presence of viral particles with full infection capability. HBV is a hidden threat for HBsAg-negative patients with hematological malignancies, born in endemic areas. In our series, about one-half of anti-HBc-positive patients harbored criptic reservoir of HBV DNA in the liver, and one out of five developed overt hepatitis B during the breakdown of immunosurveillance due to HSCT. Lamivudine treatment was successful to prevent fatal hepatic failure.

PO-201

ASSESSMENT OF THYMIC OUTPUT IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES FOLLOWING NON-MYELOABLATIVE ALLOGRAFTING

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The introduction of non-myeloablative/reduced intensity conditionings has increased the eligible age for allografting up to 65-70 years. The thymic function is fundamental for the generation of T-cell diversity following hematopoietic cell transplantation even though it is generally considered to decline with age. However, recent studies, performed in animals and humans, have shown that the thymic activity appears maintained in elderly individuals and may still play a role in T-cell reconstitution in different immunological settings. The generation of TCR diversity occurs in the thymus through the recombination of gene segments encoding the variable parts of the TCR α and β chains. During this process, by-products of the rearrangements are generated in the form of signal joint T-cell receptor excision circles (sjTRECs). sjTRECs are stable extrachromosomal DNA fragments, not replicated during mitosis and thus diluted with each cell division. They are therefore more frequent in naive T cells that have recently left the thymus. sjTRECs quantification in peripheral blood is currently considered the most reliable tool to assess thymic activity. We used quantitative sjTRECs analysis as a marker of thymic function in the immune reconstitution after non-myeloablative/reduced intensity stem cell transplantation. Thymic output was assessed on 39 patients, median age 54 (range 23-64) years, conditioned with low dose TBI (200 cGy), with/without fludarabine (90 mg/m^2 total), or cyclophosphamide-thiotepa followed by G-CSF mobilised peripheral blood stem cell infusion from HLA identical siblings or volunteer donors. Diagnoses included: multiple myeloma (no=30), acute myeloid leukemia (no=3), myelodisplastic syndrome (no=2), chronic myeloid leukemia (no=1), Hodgkin disease (no=2), and chronic lymphocytic leukemia (no=1). Brefly, genomic DNA was purified with QIAamp Blood Mini Kit (QIAgen Inc, Valencia, CA) from Ficoll separated peripheral blood mononuclear cells obtained at different time points: baseline, and, 3, 6, 12, 18 months, 2, 3, 4 and 5 years post-transplant. Real Time PCR analysis was performed with TREC specific primers to detect recent thymic emigrants: forward 5'-TGGTTTTTGTAAAGGTGCCCAC-3' (50 nM), reverse 5'-GTGCCAGCTGCAGGGTTT-3' (50nM) and the oligo 5'(FAM)CATAGGCACCTGCACCCCGTGC (TAMBRA)p-3' (250 nM) as a detection probe. The GAPDH gene was amplified to standardise DNA content. Amplification reactions (25 mcl) contained 100ng of genomic DNA extracted from patient samples or standards, TaqMan universal PCR master mix (Perkin Elmer Applied Biosystems, Foster City, CA,USA), and the appropriate primers and probes. All reactions were performed in the Model 7900 Sequence Detector using standard parameters and analysed using the GeneAmp software (Perkin Elmer Apllied Biosystems). All samples were measured in duplicate PCR reactions. TREC copies/mL blood were calculated from the number of TREC molecules in the sample and corrected with CD3 T cell counts evaluated by flow-cytometry on blood mononuclear cells. Median sjTRECs values were as follows: 115.65 (range 0-5341.9) at baseline, and 54.66 (0-3718.3), 78.1 (0-2234.7), 623.35 (0-12732), 184.65 (0-2317.4), 536.66 (0-36509), 220.82 (0-14286.1), 285.16 (0-932.6), 672.57 (0-2728.8) at 3, 6, 12, 18 months, 2, 3, 4 and 5 years respectively. Healthy donors had significantly higher sjTRECs values at the time of donation compared to recipients. Importantly, one 60-year-old leukemic patient thymectomized 10 years prior to allografting for thymoma did not show any sjTRECs in peripheral blood at baseline and after transplant thus proving that the residual thymic tissue of the other elderly patients was particularly active. Of note, sjTRECs values were significantly reduced in patients with chronic graft-versus-host disease (GVHD) strongly suggesting that thymopoiesis is suppressed by GVHD. CDR3 spectratyping analysis of the TCR repertoire is in progress at the time of this analysis as well as sjTRECs quantification on highly purified CD3/CD4- and CD3/CD8- T cells by cell sorting. In a subset of 33 patients, we also evaluated the presence of naive CD4+CD62L+CD45RA+bright T cells and of memory CD4+CD62L+ CD45R0+bright T cells. Preliminary data showed a gradual increase of these cell populations at 3 years with median values of 882/mcl (r 545-1045/mcl) and of 532/mcl (r 256-533/mcl) respectively showing a slow immune reconstitution. Overall, our results provide evidence that adult thymus activity contributes substantially to T cell immune reconstitution after hematopoietic stem cell transplantation and thus could become a future target for therapeutic intervention with agents such as recombinant human keratinocyte growth factor-1 or IL-7.

PO-202

HEMATOPOIETIC STEM CELL TRANSPLANTATION FROM HAPLOIDENTICAL RELATED DONOR FOR HEMATOLOGICAL MALIGNANCIES

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Between September 1997 and May 2005, 31 patients (M20, F11) with high-risk or refractory/relapsed hematological malignancies (ALL 11, AML 13, CML 6, HD 1) received allogeneic PBSCs from 2-3 antigens mismatched haploidentical related donors. Fourteen patients were in first (n=8) or in second (n=6) complete remission of acute leukemia and the remaining 17 patients were in more advanced phase of disease (mostly in relapse). The conditioning regimen included TBI (1200 cGy) (n= 18) or Busulphan (16 mg/Kg) (n=5) or Melphalan (140 mg/m^2) (n=8) in addition to Thiotepa, Fludarabine and ATG. Graft manipulation was performed by one step procedures of CD34+ cell selection and CD4/CD8 negative depletion using Isolex 300i (Baxter). The median dose of CD34$^+$ and CD3$^+$ cells was 9.92 (range, 5-16,1) $\times 10^6$/kg and 1,02 (range, 0,2-15,5) $\times 10^4$/Kg respectively. With this protocol the non-relapse transplant related mortality (TRM) at 6 months was 41%. The main cause of death was infection (10 out of 13 patients). Leukemia relapse occurred in 13 patients after a median of 98 days (range, 36-279). As of April 2006, 6 patients are alive and well after a median follow-up of 78 months (range, 9-104). In an attempt to ameliorate these results and in particular to decrease the TRM, we designed a new protocol in which T cell depletion was abrogated. The conditioning regimen included Cytarabine 3g/bw/day i.v. in 2 divided doses on days -7, -6 and -5, Cyclophosphamide 45 mg/Kg/day on days -5 and -4, 1000 cGy TBI from a Co60 source in 4 fractions on days -2 and -1. For patients not elegible for TBI, Treosulfan was included in the protocol at dose of 14 g/bw/day from day -4 to -2. Donors were primed with G-CSF (Filgrastim) at 3-4 microg/Kg/day for 7 consecutive days from day -7 to -1. Bone marrow cells were collected from the posterior iliac crests on the eight day with a target volume collection of 15-20 ml/Kg recipient bw and were infused fresh and unmanipulated on the same day. For GvHD prophylaxis a combination of 5 drugs was used: ATG-Fresenius at 5 mg/Kg/day from day -4 to -1; Cyclosporine at 1,5 mg/Kg/day i.v. from day -7 to -1, 3 mg/Kg/day i.v. from day 0 until bowel function recovered and then switched to oral route to 5 mg/Kg until day +365; MTX 15 mg/bw on day +1 and 10 mg/bw on day +3, +6 and +11; Mycophenolate mofetil 1 g/day in 2 divided doses from day +7 to +100; Basiliximab 20 mg i.v. on day 0 (2 h before marrow infusion) and on day +4. From July 2005 to March 2006, 4 patients with high risk AML were enrolled. All patients failed to find an unrelated matched donor in the worldwide registry over 3 months and were given the transplant option from an haploidentical related donor. The median dose of nucleated cells, CD34+ and CD3+ cells was $3{,}1\times10^8$/kg, $2{,}8\times10^6$/Kg and $22{,}3\times10^6$/Kg respectively. One patient died early of septic shock on day +8. The other 3 patients achieved full donor cell engraftment with a median time of 21 days to reach > 0.5 $\times 10^9$/L PMNs and 26 days to reach > 20×10^9/L platelets. The post-trans-

plant outcome was uncomplicated. Only 1 patient developed grade I acute GvHD. One episode of septicemia and 1 episode of CMV reactivation were observed. The 3 surviving patients are now at 9, 8 and 3 months post-transplant without chronic GvHD in excellent clinical and hematological conditions without infections. Most important, the immune system reconstitution at 3 and 6 months post-grafting seems to be similar to that observed in HLA identical marrow transplants. The small number of patients treated with the protocol as described here do not permit any final conclusion. Nevertheless, the results are promising and encouraging and in our opinion deserve further study with more patients and longer follow-up.

PO-203

SALVAGE TREATMENT WITH HIGH-DOSE THERAPY AND PBSCT IN HIGH GRADE NON HODGKIN LYMPHOMAS

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Background. The inclusion of high-dose therapy and PBSCT as salvage treatment in patients with HG-NHL is generally planned in designed protocols. However, it is generally difficult to apply the therapy to all expected patients due to several reasons, including the disease progression which leads frequently to change the assigned therapy, or to compliance of patients with high-dose therapy, or to difficult to obtain an adequate number of CD34 cells. Aims. We focus the study on a population of young patients with HG-NHL addressed to salvage treatments including high-dose therapy. The analysis is finalized to show if there are difference on survival according to an intention to treat (ITT) between those addressed to a conventional (CT) or to an high-dose (HDS) salvage treatment, either in patients relapsed or in those primarily resistant to previous therapy. Methods. Since 1988 up to 2003, 1122 patients with HG-NHL completed the assigned therapy according the undergoing protocols; 236 patients of age < 60 years were assigned to a salvage program either because primarily resistant (103=44%) or relapsed (133=56%). Based on ITT analysis we valuated CT or high-dose therapy HDS in 181 valuable patients. One hundred twenty one patients were assigned to CT (67%) and 60 to HDS (33%). Among the 60 patients addressed to HDS 38 (63%) did the planned therapy and 22 didn't, 62% were refractory and 65% relapsed. Crude results show in refractory patients 14% CR with CT and 30% with HDS; instead relapsed patients obtained 45% and 48% CR with CT and HDS, respectively. Relapse following salvage therapy, occurred in 70% and 36% of refractory patients treated by CT and HDS, respectively, and in 40% and 27% of relapsed patients treated by CT and HDS, respectively. The OS of 80 refractory patients according to ITT analysis show 8 months median survival, 6 and 10 months for CT and HDS, respectively; the 3 years survival is 22% and 32%, respectively (p=0.142). The analysis done for the therapy effectively performed show in refractory patients a median survival of 6 and 18 months for CT and HDS, respectively (p=0.056). The OS of relapsed patients is 26 months median, 21 and > 84 months for CT and HDS, respectively. The 3 years survival is 38 and 70%, for CT and HDS, respectively (p=0.029). The analysis for therapy effectively done show a better significance of survival according to two arms of treatment (p=0.025). Conclusions. Our study demonstrated that HDS in relapsed patients with HG-NHL exerts a better outcome than CT, whether the same result could be obtained in refractory patients should be further investigated by prospective studies even if a positive trend could be already disclosed in this subset of patients.

PO-204

IMBALANCE OF THE OSTEPROTEGERIN/RANK-L RATIO IN BONE MARROW MICROENVIRONMENT AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Bone loss is a common and multifactorial complication after allogeneic stem cell transplantation (allo-SCT); it is related to the transplant procedure, gonadal failure, immunosuppression, immobilization and abnormalities in calcium and vitamin D absorption and metabolism. Osteoprotegerin (OPG) is a major regulator of bone remodeling, which acts by neutralizing the receptor activator of nuclear factor-κ B ligand (RANK-L), thereby preventing RANK-L from binding to its receptor RANK. RANK-L is the primary mediator for osteoclast differentiation, function and survival, thus enhancing bone resorption; its blockade has been documented to prevent bone loss. In 36 patients and 36 controls, we investigated the relationships between BMD, circulating OPG, RANK-L, interferon-γ (IFN-γ) and IL-6 levels; in addition, OPG and RANK-L were measured in marrow plasma and in conditioned medium of long-term cultures of marrow mesenchymal-derived osteogenic cells. Lumbar and femoral BMD were lower in patients than in controls (p<0.01). Serum OPG (sOPG) and IFN-γ were significantly higher in patients than in controls (p<0.05). Patients' serum IFN-γ correlated with OPG levels (r=0.4; p=0.03). IL-6 did not differ between patients and controls. By contrast, OPG was significantly lower in patients than in controls (p<0.001) in bone marrow plasma. In both patients and controls, there was a significant increase (p<0.01) in the OPG production in the conditioned medium of mesenchymal-derived osteogenic progenitors after one (4-to-6 folds) and three months (8-to-12 folds) of culture when compared to the values in bone marrow plasma. However, OPG production in conditioned medium of cultures resulted significantly lower in patients than in controls after one (p=0.035) and three months (p=0.003). The number of CFU-O colonies was lower in patients than in donors (26.7±8 vs 74.4±29; p<0.001). RANKL levels did not differ between patients and donors either in marrow plasma or in the conditioned medium of mesenchymal-derived osteogenic progenitors. In both groups, RANK-L resulted higher in marrow plasma than in the conditioned medium (p<0.01, both groups), without any difference between one and three months of culture. The OPG/RANK-L ratio was similar in patients and controls in marrow, but resulted significantly lower in patients in conditioned media after 1 and 3 months of culture (p<0.001). There was no correlation between sOPG and marrow OPG, RANK-L levels and densitometric values. Our findings suggest that after allo-SCT: 1) sOPG bears no relationship with OPG in the bone marrow; 2) increased sOPG can be a response to factors such as inflammatory cytokines promoting bone loss; 3) low bone marrow OPG levels may be one of the causes of the persistent quantitative and qualitative deficit of osteoblastic precursors; 4) reduced OPG/RANK-L ratio in bone microenvironment may negatively affect bone remodeling.

PO-205

CD4+CD25+ REGULATORY T CELLS AND THEIR CD4+CD25- COUNTERPART EXHIBIT DIFFERENT TCR REPERTOIRE USAGE AFTER ALLOGRAFTING

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After allogeneic haematopoietic stem cell transplantation (SCT) the overall T cell receptor (TCR) repertoire is characterized by a lower diversity and a markedly skewed pattern. Its normalization may start at about 6 months after transplant but most patients continue to show an abnormal profile until 2 or 3 years. In humans 1-2% of $CD4^+$ T cells in the periphery is represented by a $CD25^+$ regulatory (Treg) subset which plays a crucial role in the maintenance of peripheral tolerance.

Although it is known that the administration of Treg cells has a protective effect in murine models of acute graft-versus-host disease, their role after SCT in humans has not been fully elucidated. Since naturally occurring Treg lymphocytes require maturation in the thymus, we assessed whether they exhibit any impairment in their TCR repertoire after transplantation, similar to what observed in conventional T cells. In particular, we explored the TCR Vβ CDR3 repertoire of $CD4^+CD25^+$ Treg cells, focusing on its overall complexity and on the degree of similarity to CD4+CD25- conventional T (Tconv) cells. We analyzed 10 patients who had received SCT for chronic myeloid leukemia. After CD4+CD25+ and CD4+CD25- cell isolation, RNA extraction and reverse transcriptase PCR, CDR3 region fragment analysis was performed through capillary electrophoresis. Conventional spectratyping evaluation was carried out by calculating an overall complexity score and by determining the percentage of skewed and oligoclonal Vβ profiles. Moreover, we developed a new analysis method to quantify the proportion of Vβ subfamilies with similar profile between the Treg subset and its Tconv counterpart. Although we observed a significantly higher percentage of skewed and oligoclonal Vβs in both cell subpopulations in patients less than 3 years after SCT, the conventional analysis systems showed essentially similar TCR patterns between Treg and Tconv cells. We then compared the spectratyping profiles of the 2 cell subsets within each Vβ subfamily in each subject. As a tool we developed a new *similarity score*, expressing the proportion of Vβs with similar profile between Treg and Tconv subsets. We detected a positive correlation between similarity score and time after SCT (Pearson correlation coefficient=0.65). A higher score was observed in patients more than 3 years after allografting (mean 0.90 vs. 0.61, p=0.01). Noticeably, in patients less than 3 years after SCT the differences were very often ascribable to the detection in the same Vβ subfamily of an oligoclonal profile in the Tconv but not in the Treg subpopulation. This specific pattern was almost exclusively confined to this group of patients (mean 52% vs. 5%, p=0.002). Our data show that the repertoires of Treg and Tconv cells exhibit significant differences early after SCT, while they tend to become identical with full reconstitution. These differences are mainly ascribable to Vβ subfamilies expressing an oligoclonal profile in the Tconv but not in the Treg subset and could either reflect a discrepancy in the *in vivo* reactivity against common antigenic stimulations or be the result of a different post-transplant ontogeny.

PO-206

TELOMERE LENGTH IS PERMANENTLY REDUCED IN MYELOID CELLS FROM LONG-TERM SURVIVORS LYMPHOMA PATIENTS FOLLOWING HIGH DOSE CHEMOTHERAPY AND AUTOGRAFT

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Backgrund. Telomere undergoes progressive shortening at each cell division and therefore it can be considered a good marker of cell replication history. During post-autograft hematopoietic recovery, stem and progenitor cells undergo increased replicative proliferation and this can result in abnormal telomere loss at least in the early period after autologous bone marrow transplantation. *Aims.* Aims of the study were to evaluate hematopoiesis at long-term following high-dose chemotherapy and peripheral blood stem cell (PBSC) transplantation; moreover to better understand if telomere loss observed during the early period after autograft is transient or stable even after several years. To answer these questions we investigated: i. telomere length (TL) in peripheral blood (PB) and bone marrow (BM) cells; ii. *in vitro* functional characteristics of both stem and progenitor cells. *Patients and Methods.* Samples of Peripheral Blood (PB) and Bone Marrow (BM) obtained from 31 subjects (12 female and 19 male) were analised. All patients were in continuous complete remission with a median follow-up of 5.8 yrs. (1 to 11 yrs.) since autograft. All subjects were autografted with large quantity of PBSC (median CD34+ve cells/kg: 7×10^6). Their median age at the time of transplant was 44 yrs. (range 18-65). TL was determined by Southern Blot analysis on mononuclear cells (MNC) from PB and BM samples and on separated PB granulocytes (GN). BM progenitor cells were investigated by the *in vitro* culture assays and both committed (CFU-GEMM, CFU-GM, BFU-E) and immature (LTC-IC) progenitors were evaluated. RESULTS: TL analysis on separated PB granulocytes showed significantly shorter values in autografted subjects compared to age-matched healthy controls (p<0.05) (Figure A). A similar behavior was observed on BM cells, while no significant differences were observed when TL was assessed on PB MNC (*data not shown*). Telomere length reduction appeared to be stable, with no significant difference in the extent of telomere loss between subjects observed at different time-periods since autograft (Figure B). Both immature and committed BM progenitors were found to be markedly reduced compared to normal controls. *Conclusions.* i. telomere length is reduced in myeloid cells from subjects surviving up to 10 years following autograft; ii. telomere length reduction observed during the early period following autograft is permanent in spite of the large amounts of transplanted CD34+ve cells; iii. a marked reduction of BM immature and committed progenitors is also maintained at long-term. Thus high-dose chemotherapy and PBSC autograft may result in myelopoietic cell abnormalities that appear to be irreversible.

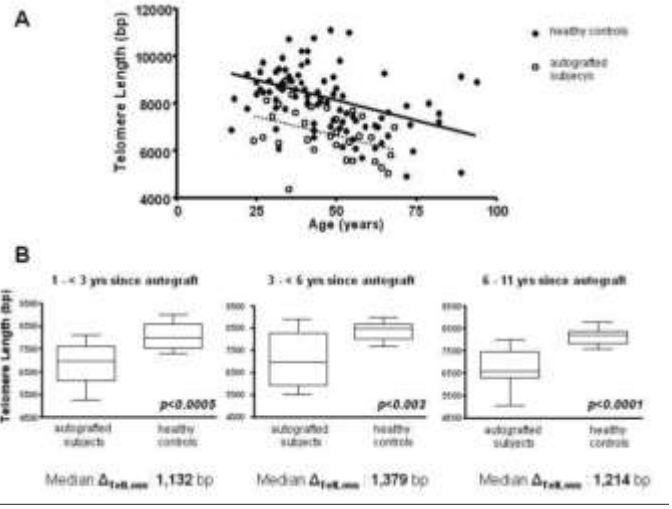

Figure.

PO-207

TELOMERE LENGTH OF HEMATOPOIETIC CELLS FOLLOWING PERIPHERAL BLOOD PROGENITOR CELL TRANSPLANTATION REFLECTS THAT OF GRAFTED STEM CELLS

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Background. In human somatic cells, telomeres decrease at each mitotic division and progressively shorten with increasing age. Due to this peculiarity, telomere restriction fragment (TRF) length has been used as a marker of cell aging. Shortening of telomeres could contribute to hematopoietic stem cells (HSCs) senescence, especially when HSCs are stressed under certain non-physiological conditions (i. e. bone marrow reconstitution after high dose (hd) chemoterapy or HSC transplantation). In a previous study (Ricca I et al, Leukemia 2005) we analysed TRF length on peripheral blood progenitor cells (PBPC) harvested after two tightly-spaced hd-chemotherapy courses, i.e. hd-cyclophosphamide (CY) and hd-Ara-C, and found that PBPC TRF length markedly shortened at the second hd-course. *Aim of the study.* To assess how TRF length of the graft may influence telomere status of post PBPC transplantation hematopoiesis. *Patients and Methods.* TRF length was monitored in 26 patients undergoing autograft with PBPC collected after hd-CY (13 patients) or hd-Ara-C (13 patients). Overall, their median age was 44 years (range 23-60). TRF length was assessed both on grafted material and on bone marrow (BM) samples taken at a median time of 24 months after transplant. All patients were in complete remission of their underlying disease at the time of TRF length assessment. As control group, we analysed 8 selected allografted patients in which donor PBPC had TRF

length significantly longer than recipient BM cells. TRF length was evaluated by Southern blot, as previously described (Ricca I *et al.,* Leukemia 2005). *Results.* As expected, TRF length was markedly shortened in post-Ara-C compared to post-CY PBPC. Overall, median TRF length in post-CY PBPC was 8100 (range 6105-11024) while in post-Ara-C PBPC was 7046 (range 5561-8906) (p<0.0001). Interestingly, at post-transplant follow-up, we found that while median TRF length in patients autografted with post-Ara-C PBPC was 7092 bp (range 5851-8227) (p=NS compared to pre-transplant value) (Figure, panel A), patients autografted with post-CY PBPC had a significantly longer post-transplant TRF length (7474 bp, range 6707-8808) compared to pre-transplant value (p<0.005) (Figure, panel B). Moreover, we found the same in the allografting setting. Indeed, patient post-transplant TRF length was similar to the donor cells (p=NS) but markedly longer if compared to patient pre-transplant value (p=0.01). *Conclusion.* TRF length of BM cells following PBPC transplant reflects that of graft, suggesting that telomeres of hematopoietic cells can be modified according to telomere length of grafted stem cells.

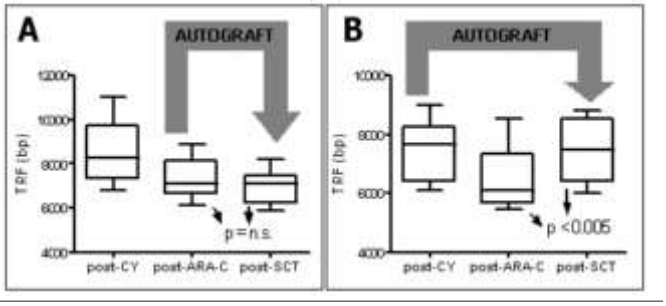

Figure.

Stem Cell Transplantation II

PO-208

AUTOLOGOUS PERIPHERAL STEM CELLS IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION: A SINGLE INSTITUTION EXPERIENCE

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Background. Recent studies suggest that bone marrow–derived progenitor cells induce both vasculogenesis and angiogenesis in experimental models of myocardial infarction. Implantation of autologous bone marrow stem cells into the infarcted myocardium is a novel therapeutic approach to repair heart damage and restore myocardial function after acute myocardial infarction. However, only a double-blind randomized clinical trial can assess the balance between benefit and theoretical risk of such approach. Aim of the study. We analyzed the expression of some humoral factors and the trafficking of early and late endothelial progenitor cells in a setting of myocardial infarction patients who underwent intramyocardial injection of autologous bone marrow mononuclear cell according to a double-blind randomized phase-II clinical trial. *Patients and Methods.* The study started in March 2004; a total of 37 patients who had suffered from acute transmural myocardial infarction less than 6 months before admission, were recruited for the study. The patients were randomized 1:1 to receive bone marrow mononuclear cell or placebo implantation plus conventional coronary artery bypass grafting. Serial samples of peripheral blood mononuclear cells, serum and plasma were collected to evaluate circulating stem cell subset-populations and to examine factors potentially involved in the homing and retention of stem cell in the myocardium. *Results.* No major perioperative complications were observed. One patient died of non-transplantation related cause four months after bypass surgery. In our study the large majority of patients showed a significant improvement both in left ventricle dejection Fraction and in left ventricle volumes. After intervention, the number of circulating hematological CD34+ cells coexpressing either CD133 or CD117, early CD34+/VEGFR-2+ and late CD34+/CD31+ endothelial cell subsets, circulating mononuclear cells expressing CD11b and CD62 adhesion molecules, increased at different time points going back to baseline value. Systemic vascular endothelial growth factor -2 and stromal cell-derived factor-1 α were quickly up-regulated. *Conclusion.* From our experience certain interim conclusions can be drawn. One, adjunctive bone marrow-derived cell implantation at the time of bypass surgery appears to be feasible and safe. The second conclusion is that both cell and placebo infusion in infarcted patients could regulate endothelial progenitor cell mobilization and angiogenic/homing factors which might act to recruit circulating stem cells to the ischemic heart. Actually, we are not able to provide any definitive conclusion on the association between biological results and clinical outcome since this study is still blinded. In agreement with the protocol, the first 20 patients datas has been subordinates to an analysis ad interim, that will be illustrated during conference.

PO-209

IMMUNE RECONSTITUTION OF THE T CELL COMPARTMENT BY FLOW CYTOMETRY FOLLOWING ALLOGENEIC NON MYELOABLATIVE HEMATOPOIETIC CELL TRANSPLANTATION IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

Gilestro M, Omedè P, Bruno B, Spagnolo M, Ferro F, Ruggeri M, Brunetti M, Caltagirone S, Di Bello C, Cimolin L, Sfiligoi C, Cena S, Giaccone L, Rotta M, Sorasio R, Boccadoro M

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Allogeneic stem cell transplantation is a potentially curative therapy for a variety of hematological malignancies. However, relapse and treatment-related toxicity are major obstacles to cure. Reduced-intensity/non-myeloablative conditioning were designed to initially establish mixed-hematopoietic chimerism and to serve as a platform for additional cell immunotherapy aimed at eradicating tumor cells in elderly patients (up

to 70 years). Despite this progress, the risk of post transplant infections and graft-versus-tumor effects rely on residual thymic function, notoriously reduced in adults. Post-transplant T cell reconstitution follows two pathways: one in the immediate post-transplant period, is thymus independent and characterised by transplanted donor T cells with a limited T cell receptor repertoire; the other,is thymus dependent and leads to the selection of new T cell clones with a wider repertoire that strongly affect the long term reconstitution. We evaluated by flow cytometry the immune recovery in 55 patients with hematological malignancies, median age 51 years (range 34-64), 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. The immunophenotypic profile was assessed on blood samples at different time points: baseline, and at 28, 56, 84, 100, 180 days, and at 1, 2, 3, 4, 5 years post-transplant. Briefly, fresh samples were stained with anti-CD3FITC/CD4PerCp, anti-CD3FITC/CD8APC, anti-CD3FITC/CD16+ CD56PE, anti-CD19APC, anti-CD20PE, anti-CD45RAFITC/ CD45R0PE/ CD4PerCp/CD62LAPC,anti-CD45RAFITC/CD45R0PE/CD8PerCp/ CD62LAPC, and hemolysed with a NH4Cl solution. Eighty-thousand events were acquired on a FacsCalibur (Becton Dickinson), and analysed with CellQuest Pro software. Blood CD3+CD4+ T cell counts >200/µL promptly recovered by day 28 with median values of 291/µL (range 16-787/µL), gradually increasing to 488/µL (range 140-1119/µL), 506/µL (range 261-1693/µL), and 715/ µL (range 296-1061/µL), at 18 months, 2 and 3 years, respectively. Naive CD4+CD45RA+bright T cells gradually increased to values of 51/µL (range 1-299/µL), 42/µL (range 0-211/µL), 64/µL (range 3-260/µL), 69/µL (range 6-258/µL), 100/µL (range 18-154µL), 102/µL (range 2-386/µL), and 117/µL (range 32-145/ µL), at day 28, day 180, and at 1, 2, 3, 4, 5 years, respectively. Memory CD4+ CD45R0+bright remained stable with median values of 153/µL;(range 6-335/µL) and 183/µL (range 0-538/µL), by day 28 and day 100, respectively; then gradually increased to values of 224/µL (range 1-845/µL), 314/µL (range 2-839/µL), 535/µL (range 139-729/ µL), 462/µL (range 0.1-922/µL), and 514/µL (range 2-571/µL), at 1, 2, 3, 4, 5 years. In a subset of patients, the evaluation of the co-expression of the CD45 isoforms showed that the number of CD4+CD45RA+CD45R0+ T cells reached median values of 65/µL (range 1-382/µL) by day 28 and 82/µL (range 7-1001/µL) at 12 months, respectively; then gradually increased to median values of 131/µL (range 46-1435/µL), 158/µL (range 58-341/µL), 181/µL (range 35-499/µL), and 286/µL (range 93-712/µL), at 2, 3, 4, 5 years. Blood CD3+CD8+ T cells counts reached median values of 158/µL (range 15-563/µL) by day 28, increasing to 373/µL (range 91-1926/µL), 746/µL (range 23-1648/µL), 923/µL (range 433-1668/µL), and 1049/µL (range 772-1630/µL), at day 100, at 1, 3, 5 years, respectively. CD4/CD8 ratio was 1.8 (range 0.2-6.7) by day 28, decreased to 0.7 (range 0.2-3.8) by day 100, and remained low at values of 0.72 and 0.79 at 2 and 5 years, respectively. In a subset of 33 patients we evaluated the presence of naive CD4+CD62L+ CD45RA+bright T cells and of memory CD4+CD62L+CD45R0+bright T cells. Preliminary data showed an increase of these cell populations at 3 years with a median value of 882/µL;(range 545-1045/µL), and 532/µL (range 256-533/ µL), respectively. Our findings suggest a slow immune reconstitution during the first two years post-transplant that differs from normal T lymphocyte ontogenesis. The quantitative analysis of TRECs currently in course and the analysis of very naive T cells and very memory T cells by CD62L expression will allow to quantify the residual thymic function in this group of elderly patients.

PO-210

ACUTE GRAFT-VERSUS-HOST-DISEASE AND TREATMENT-RELATED MORTALITY IN PATIENTS UNDERGOING AN ALLOGENEIC PERIPHERAL BLOOD STEM CELL TRANSPLANTATION ARE INFLUENCED BY B-CELL CONCENTRATION IN THE GRAFT

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The majority of allogeneic stem cells transplants are at present performed with donor derived peripheral blood stem cells (PBSC) after mobilization with granulocyte-colony stimulating factor. The effects of the cellular composition of the graft on the outcome of these patients have so far not been discerned. Aim of this study was to correlate the concentration of nucleated cells, mononucleated cells, stem cells, T cells, B cells and NK cells in the graft with the clinical parameters of patients who have undergone an allogeneic PBSC transplantation from an HLA identical sibling. We performed the analysis on the grafts infused to 63 consecutive patients (27 AML, 10 ALL, 13 CML and 13 MM) between November 1999 and November 2005. The conditioning regimen was Busulphan 16 mg/kg of body weight (BW) plus Cyclophosphamide 120 mg/kg BW (AML, CML and MM patients) or TBI 12 Gy plus Cyclophosphamide 120 mg/kg BW (ALL patients). Cyclosporine plus Methotrexate were given to all patients for GVHD prophylaxis. Hemochromocytometric analysis and three-color immunofluorescence were performed on the apheretic samples, using antibodies against CD3, CD4, CD8, CD20, CD16, CD56 and CD34. Actuarial curves for overall survival (OS), leukaemia-free survival (LFS) and event-free survival (EFS), have been calculated according to the Kaplan-Meier method; cumulative incidence has been used to evaluate the probability of engraftment, acute and chronic graft-versus-host-disease (GVHD), relapse and treatment-related mortality (TRM); finally, the correlation with the number of cells and cellular subsets infused has been calculated by means of the correlation coefficient of Spearman. All the variables for which a p-value <0.1 was recorded underwent a multivariate analysis using the proportional model of Cox. The median number of infused cells/Kg of the recipient BW was: nucleated cells 10.9×10^8 (range 5-51); mononucleated cells 6.7×10^8 (range 1.56-21); CD34 5.9×10^6 (range 4.07–14); CD3/CD8 0.9×10^8 (range 0.19-3.1); CD3/CD4 1.5×10^8 (range 0.4–8.1); CD3 2.6×10^8 (range 0.07–11.30); CD20 0.28×10^8 (range 0.06–2); CD16/CD56 0.20×10^8 (range 0.01–1.7). Grade I-IV acute GVHD was observed in 24 patients (38%). In multivariate analysis, acute GVHD correlated with the disease (p 0.002), with the phase of the disease at the time of transplant (p 0.047) and with the more than median dose of CD20$^+$ infused cells (p 0.06). Also TRM correlated significantly with the high concentration of CD20+ cells in the graft (p 0.02). The results of this study suggest that the concentration of B cells in the graft may predict the incidence of acute GVHD and TRM in patients undergoing an allogeneic PBSC transplantation, thus suggesting possible new preventive and therapeutic strategies in the clinical management of these patients.

PO-211

INCREASED EXPRESSION OF CD86 AND CXCR4 IN CD14+ DENDRITIC CELL (DC) PRECURSORS IN PERIPHERAL BLOOD (PB) AND BONE MARROW (BM) OF PATIENTS WITH CHRONIC GVHD

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Recent studies in animal models have shown that chronic GVHD requires persistent host antigen presentation by donor antigen presenting cells (APC). In this study we analyzed the number and phenotypic characteristics of circulating APCs in patients with and without chronic GVHD (cGVHD) after allogeneic hematopoietic stem cell transplantation (HSCT). Flow cytometry analysis was performed on fresh PB (n=19) and BM (n=7) samples of patients with cGVHD. As control, we analyzed PB (n=12) and BM (n=15) samples of patients without cGVHD and at comparable time after transplant. Each sample was stained to detect BDCA-1+ myeloid DC (mDC), BDCA-2+ CD123+ plasmacytoid DC (pDC) and CD14$^+$ monocytes, as well as the expression of adhesion receptors (CD62L and CD49e), costimulatory molecules (CD80, CD86 and CD40) and chemokine receptors (CXCR3, CXCR4, CCR5 and CCR7) on the surface of APCs. Patients with cGVHD showed increased numbers of CD14$^+$ cells in the BM (p=0.006), but not in the PB (p=0.09), and similar numbers of mDC and pDC, as compared to patients without cGVHD. CD14$^+$ cell populations were then compared in the two groups, and in patients with cGVHD a greater Mean Fluorescence Intensity (MFI) of CD86 was observed both in BM (p=0.02) and PB (p=0.04). A trend for an increased expression of CXCR4 chemokine receptor was also observed in PB monocytes (p=0.06) of patients with cGVHD. Analysis of these markers in mDC and pDC showed no correlation with cGVHD. Interestingly, in patients with cGVHD who were analyzed after treatment with prednisone >1 mg/kg, the expression of CD86 on monocytes was significantly reduced both in BM (p=0.02) and PB (p=0.04). This study demonstrates that in patients with cGVHD: there is a greater number of CD14$^+$ monocytes in the BM; both in BM and PB CD14$^+$ monocytes are more activated with higher expression of CD86 and

CXCR4; treatment with prednisone significantly downregulates the expression of these receptors. Since a quantitative and qualitative difference in circulating $CD14^+$ monocytes, but not mDC or pDC, is observed in patients with cGVHD, we hypothesize a role of donor $CD14^+CD86+$ DC precursors in the pathogenesis of cGVHD.

PO-212

CHRONIC GVHD AND SEQUENTIAL STUDY OF CYTOKINE PATTERNS AND LYMPHOCYTE SUBSETS IN PATIENTS UNDERWENT ALLOGENEIC STEM CELL TRANSPLANTATION WITH REDUCED INTENSITY CONDITIONING

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Chronic GVHD (cGVHD) is the most common and severe late complication after allogeneic stem cell transplantation (SCT), especially with reduced intensity conditioning (RIC). Impaired immune reconstitution and unbalanced Th1-type or Th2-type immune response with citokines dysregulation play a key role in the pathogenesis of chronic GVHD (cGVHD). In order to study the immunologic mechanisms involved in cGVHD, we evaluated the Th1 (TNF-α, IFN-γ) and Th2 (IL-4, IL-6, IL-10) cytokine patterns and the T-B-NK lymphocyte subsets in 8 healthy donors and in 12 patients undergoing allogeneic SCT-RIC (9 patients with related and 3 patients with unrelated donor). We assessed by ELISA the serum levels of TNF-α, IFN-γ, IL-4, IL-6, IL-10 and soluble tumor necrosis factor receptors I and II (sTNF-R). Serum levels were assessed before transplantation, weekly during the first month and monthly from second to twelfth month after SCT. Total lymphocytes and their subsets with different costimulatory molecules (CD4, CD8, CD19, CD28/3,CD25/4, CD134/4, CD152/3, CD16/56, CD4/45ro, CD4/45ra, CD8/45ro, CD8/45ra) were evaluated by flow citometry in peripheral blood (PB) monthly after SCT. Cyclosporine A was used as GVHD prophylaxis in all patients from third month until its tapering or until the beginning of the therapy against extensive cGVHD. Nine out of 12 patients developed cGVHD (6 extensive) at a median time of 6 months (range, 5-10 mo). The levels of cytokines did not differ significantly in patients pre-SCT and healthy donors. Patients with cGVHD differed from those without cGVHD because of: 1) significantly higher levels of TNF-α from third to sixth month after SCT (3rd mo, p=0.01; 4th mo, p=0.03; 5th mo, p=0.01; 6th mo, p=0.04); 2) significantly higher levels of sTNF-R I at 3rd (p=0.01) and 4th month (p=0.04); 3) significantly higher levels of IL-10 (p= 0.03) and IL-6 (p=0.04) at 4th month; 4) a trend toward a lower number of NK cells in PB at 6th month (p=0.05); 5) a trend toward a lower number of CD134/4 cells (p=0.06) and CD 152/3 cells (p=0.07) in PB at 3rd month. The number of CD4/25 regulatory T cells in PB did not differ between patients with and without cGVHD. Our sequential study showed: 1. increased levels of Th1 (TNF-α) and Th2 (IL-10, IL-6) cytokines with different kinetics after SCT and before the onset of cGVHD; 2. a decrease of NK and T cells with regulatory molecules such as CD134 and CD152 after SCT and before the onset of cGVHD. These results did not show a clear and continuous prevalence of Th1 or Th2 immune response in patients with cGVHD. A Th1 or Th2 cytokine hyperproduction and different lymphocyte subsets could fluctuate from SCT to the onset of cGVHD. Further studies including more patients are required to support these preliminary results.

PO-213

REDUCED INTENSITY CONDITIONING (RIC) IN ALLO-BMT FOR RESISTANT-RELAPSED LYMPHOMAS

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Background. The use of RIC conditioning regimen is now largely applied in conditioning regimen for TMO in haematological malignancies however no definite indications exist on which patients may be benefited by this procedure although most of experiences are focused on patients with lymphomas resistant or relapsed to other therapies. Aims. To disclose if RIC may be a therapeutic option as salvage treatment with acceptable results in a population of relapsed-resistant patients with lymphomas, mostly already treated by high-dose therapy and PBSCT. The inclusion in the RIC procedure of those patients with age over 60 years leads to valuate if the toxicity, namely related to TRM, is acceptable. Methods. We present a cooperative experience on RIC focused on 42 advanced, relapsed and resistant lymphomas to several therapies including high dose and autologous PBSCT. The mean age of patients was 49 years (23-67 years). RIC consisted in the combination of TT 10 mg/Kg, Fludarabine 50 mg/×5 and CTX 300 mg×5 for 27 patients (64%) and other similar combinations for the other patients. Diseases included 13 HD resistant, 9 HGBL, 5 FL, 6 HGTL, 4 CLL, 2 ALC and 3 LPL relapsed and resistant to a series of therapies. Thirty-one patients have been already treated by high-dose therapy and PBSCT. Results. Thirty-six patients (86%) are valuable for response, 6 patients died within 3 months from RIC for causes not related to disease progression (TRM=14%). The response valuated within 6 months from RIC showed 26 pts in CR (72%) and 10 patients with persistence of disease (28%), 3 relapses (14%), were registered following 12, 18 and 24 months from RIC. Eight patients died (22%) for disease progression among valuable pts, one further death was registered for chronic GVHD 18 months following RIC accounting at 15 the total number of deaths including TRM (36%). Five patients are alive with lymphoma and 22 alive in CR (52%) at a mean follow-up of 28 months (7-59 months). The incidence of GVHD was registered in 16 patients (38%) and 6 of them had grade 3-4. This was not correlated to previous therapy or to type of RIC conditioning regimen or to the age of patients. Deaths accounted 11 on 31 (35%) patients who received autologous PBSCT and 4 on 11 (36%) on those who did not. Conclusions. RIC transplantation provides an high rate of remissions in patients with advanced lymphoma and an acceptable TRM. The results are independent from the previous therapy and type of disease; the incidence of GVHD and relapses are not correlated to different conditioning regimen. Future prospective trials including RIC transplantation are planned.

PO-214

INTERMITTENT NON-INVASIVE VENTILATION IN IMMUNOCOMPROMISED PATIENTS WITH ACUTE RESPIRATORY FAILURE

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Avoiding intubation for acute respiratory failure is a major goal in the management of immunocompromised patients (pts), with haematological malignancies. Patients transferred to the intensive care unit and undergoing to endotracheal intubation and mechanical ventilation are at highest risk of death. The early use of intermittent non-invasive ventilation (NIV) during acute respiratory failure can help to avoid the need for endotracheal intubation by improving patients outcomes. We conducted a study of efficacy on intermittent NIV, in 30 haematological pts at an early stage of hypoxemic acute respiratory failure according to an approved protocol. In these 30 pts, immunosuppression associated or not with neutropenia, was related to intensive chemotherapy (n=12, 40%), autologous (n=4, 13%) or allogeneic (n=8, 27%) HSCT transplantation (n=12, 40%) or it was a result of intensive corticosteroid therapy (n=6, 20%). At the onset of the respiratory complication, the patients showed: pulmonary infiltrates, fever, severe dyspnea at rest, a respiratory rate of more then 30 breaths per minute and a ratio of the partial pressure of arterial oxygen (PaO2) / fraction of inspired oxygen (FiO2) of less than 200. NIV was delivered to the pts trough a helmet. The helmet was adjusted and connected to a *Venturimeter* with a pressure support of oxygen. According to our protocol we made a basal test to the patients: after we have obtained an oxygen saturation of 94% with maximal PEEP (Positive end-expiratory pressure) and FiO2, we decreased each other the value as less as attainment of 94% oxygen saturation. So, according to the response, we decided the schedule and the time of treatment, dividing pts in 4 categories:Poor, Minimal, Mild and Good responders. For each of these categories the schedules of treatment was differentiated as: continous therapy (for Poor responders); four hours time four every days (for Minimal responders);two hours time three every day (for Mild responders); two hours time two every day (for

Good responders). The mean duration of NIV was 8 days (range 6-14). During NIV, major events (pulmonary oedema, pnx, haemoptysis) occurred in 4 pts (13%) and according to the protocol NIV was stopped. NIV was temporary discontinued for the occurrence of minor events (hypercapnia, hyperpyrexia,anxiety) in 11 cases (37%). Twenty four pts (80%) overcame the respiratory failure. Seventeen pts (57%) were discharged; 2 pts died for MOF, 3 pts died for progressive disease, 8 pts died for acute distress respiratory. In our study the intermittent use of NIV was possible without transferring the patients in intensive care unit and it permitted to avoid endotracheal intubation, with encouraging results.

PO-215

CHIMERISM ANALYSIS FOLLOWING ALLOGENEIC STEM CELL TRANSPLANTATION

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With the engraftment of allogeneic transplantation, the patient becomes a real chimera, because of the cohabitation in the same person of a genetic patrimony coming from two different people: the patient (pt) and the donor. The periodic control of chimerism is very important for several reasons. It identifies the cellular population's type present after transplantation, it allows the right somministration of immunosuppressive terapy, supporting the reaching of engraftment, and the timely individuation of a possible disease's relapse.We have investigated the kinetics of engraftement in 133 pts with different malignancies. Seventy nine (median age:47 range 22-62) received reduced conditioning regimens: 22 Flu/Mel, 26 Flu/Cy and 29 Flu/TBI and 54 pts (median age:33 range 10-58) received myeloablative conditioning regimen.We have also evaluated if CD34 cell dose influence engraftment. Due to its high sensitivity, chimerism's valutation is performed using multiplex PCR coamplification of 16 Short Tandem Repeat loci in a single reaction. Donor/recipient cell population ratio was detected by calculing peak area of PCR products for each informative marker. The median number of informative alleles was 6 (range 3-9). We have evaluated the number of patients that have reached the complete chimerism (CDC≥95% donor's cell) at days +15, +30, +90, +180, +270, +360 and so on. In the subgroups of pts that received non myeloablative conditioning regimens the outcame was respectively: 20/79 (25%), 30/79 (38%), 49/79 (62%), 69/79 (87%) 74/79 (94%).We have effectively show that engraftment's kinetics of non myeloablative transplantation is more gradual in time compared to the myeloablative transplantation. In this last one, the engraftment is more rapid and the complete chimerism is already reached on the 30th day. In non myeloablative transplantation, donor engraftment was evaluated at day +15, +30, +90 and so on, in three subgroups of pts that have received different CD34 cell dose:$<2\times10^6$/kg; $>2<8\times10^6$/kg; and $>8\times10^6$/kg. At the day +15 the kinetics of engraftment resulted significantly correleted to dose (p=0.028), while from the day +30 it didn't significantly differ in the three subgroups (p>0.5). *Conclutiosions.* The valutation of the transplantation' s kinetics of engraftment has shown that in non myeloablative transplantation is normal to have a mixed chimerism with tolerance host versus transplantation and transplantation versus host, for this reason we saggest the importance of the periodic control of chimerism in order to modify the immunosuppressive terapy in favour of the engraftmen and to identify immediately the desease's relapse. The CD34 cell dose has a noticeable effect only in the early kinetics donor chimerism (1 to 15 days).

PO-216

SUBSETS OF CD34+ AND ENGRAFTMENT KINETICS IN ALLOGENEIC PERIPHERAL STEM CELL TRANSPLANTATION

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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 23 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 36% for CD34+/CD33-, 60% for CD34+/CD38-, 5% for CD34+/DR- and 70% for CD34+/CD133+; this translates into a median dose of 1.6 x 10(e)6/Kg (range 0.3-5) for CD34+/CD33-, 2.6×10^6/Kg (range 0.1-6.2) for CD34+/CD38-, 0.4×10^6/Kg (range 0.1-2.3) for CD34+/DR- and 0.95×10^6/Kg (range 0.6-2.3)$\times10^6$/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 =-0.76, p<0.05) and 100×10^9/L platelets (r=-0.71, p<0.05); this correlation was similar to total CD34+ cells dose and neutrophil (r=-0.71, p<0.05) and platelets engraftment (r = -0.68, p=0.06). No correlation was found between the other CD34+ subsets and neutrophil and platelets engraftment. With regard to the threshold dose for early neutrophil engraftment, all 12 patients who received more than 1×10^6/Kg of CD34+/CD133+ had a neutrophil count higher than 1.0×10^9/L at 12 days. 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.

PO-217

WHAT IS THE BEST CONDITIONING FOR AUTOTRANSPLANT IN AML PATIENTS?

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The conditioning regime more used in bone marrow transplantation, in patient with AML, has been the BuCy-2. Such treatment, mieloablative and immunosuppressive, he favours the engraftment of the marrow in the allogeneic transplant, not clear the advantage in the autologous transplant. In the last years, new conditioning regimes to more intense mieloablation has been used in AML autotransplant patients. We have retrospectively evaluated, in terms of efficacy and results, the classical conditioning BuCy-2 with a conditioning with busulfan (4 mg/Kg days -5-3) and melphalan (140 mg/m^2 day -2) (BuMel). From June 2001 to December 2005 we have autotransplantated 30 patients with AML in first CR (11 males and 19 females; median age: 44 years (range 14-61) subtype FAB: M0: 2; M1: 5; M2: 9; M4: 13; M5: 1). 14 patients have been conditioned with BuMel (5 M and 9 F; median age: 44 years (range 18-61) subtype FAB M1: 3; M2: 5; M4: 5; M5; 1) and 16 with BuCy-2 (6 M and 10 F; median age: 41 years (range 14-59) subtype FAB M0: 2; M1: 2; M2: 4; M4: 8). The factors of risk in the 2 groups are similar. High risk: 4 patients (2 in every group), intermediate risk: 22 (12 in BuCy-2 and 10 in BuMel) and low risk: 4 (2 in every group). The PBSC has been the source of the stem cells in all patients, and the median CD34 infused cells has been of 5,15 and $5,2\times10^6$/Kg in BuCy-2 and BuMel groups respectively. All patients have achieved a full haematological recovery. The median days to neutrophil> 1000/mm^3 and platelets> 20000/mm^3 have been of 14 and 12 days in the BuCy-2 and BuMel groups respectively. In the BuMel group one patient is died for mycosis (mucor) at day +27 after transplantation. With median follow-up of 12 months (range 3-60 months), after autotransplant, 12 patients (85%) they are alive (9 in CR) in BuMel group; in the BuCy-2 group the median DFS and OS are 9 and 12 months respectively (Figure 1-2). The EFS projected to 33 months is 58% and 33% (Figure 3) in BuMel and BuCy-2 groups respectively, this difference is not statistically significant (p:0.04, Cox

F-test). In conclusion it is evident the advantage of a conditioning regime more mieloablative, in AML autotranpsplant patients, such difference statistically is not significant for the short follow-up and low number of patients. Is necessary a large cohort and a randomized study to confirm these preliminary data.

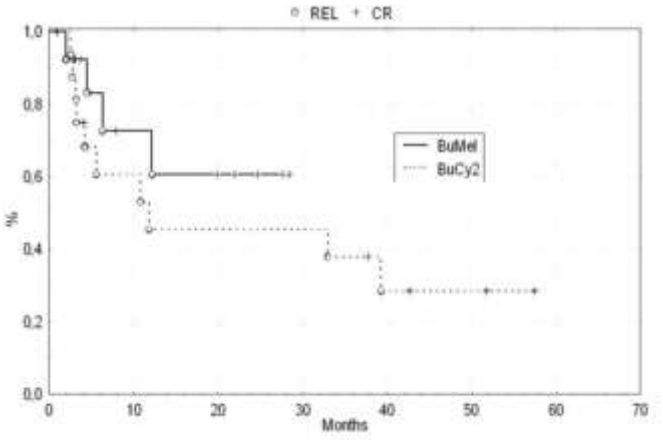

Figure 1. DFS in AML patients autotransplanted (Kaplan-Meier).

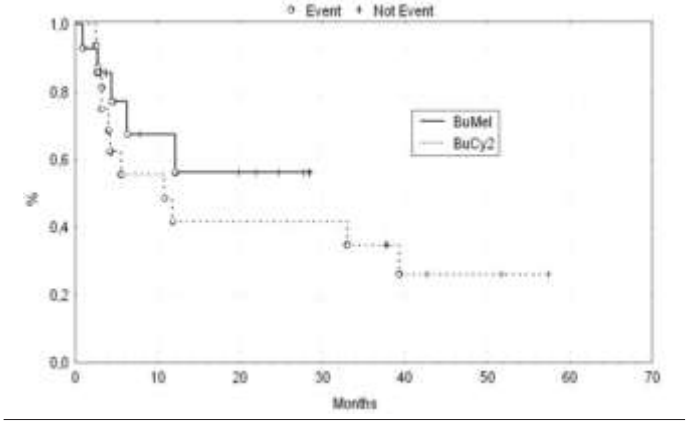

Figure 2. EFS in AML patients autotransplanted (Kaplan-Meier).

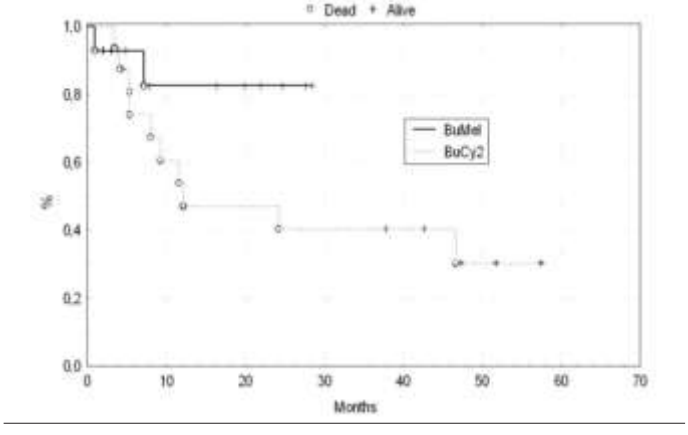

Figure 3. OS in AML patients autotransplanted (Kaplan-Meier).

PO-218

OXIDATIVE STRESS PARAMETERS IN HEMATOLOGIC PATIENTS AND THEIR ROLE AS SEVERITY MARKERS OF INFECTIOUS COMPLICATIONS

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High-dose chemotherapy and hematopoietic stem cell transplantation (HSCT) are effective therapies for haematopoietic malignancies. Hematologic diseases can cause an alteration in oxidative status (defined as balance between Reactive Oxygen Species,ROS, production and Total Antioxidant Capacity, TAC). Infections represent the most common life-threatening complications in hematologic patients and no prognostic factors are available to estimate their severity. Infections can modify oxidative status, and few data are available on oxidative stress occurring in hematologic patients. In this study we analysed the oxidative status throughout the normal course of the haematologic disease and during infectious complications. ROS levels and TAC were prospectively evaluated in 15 patients (8M/7F; aged 25-65, median 53 years) treated with HSCT (6 autologous transplants, 4 allogeneic transplants) or high-dose chemotherapy according to each patient's specific disease (4 multiple myeloma, 4 acute lymphoblastic leukemia, 3 acute myeloid leukemia, 2 mycosis fungoides, 1 chronic lymphocitic leukaemia, 1 lymphoma), and compared with ROS levels and TAC in 52 healthy controls. Blood samples were collected on admission, before, during and after chemo/radiotherapy. Baseline and Zenith/ Nadir values in ROS and TAC values were evaluated during 18 infectious complications at two days intervals until resolution. Serum ROS and TAC levels were determined by spectrophotometric methods (dROMS and OXY-Adsorbent test, Diacron, Italy, respectively). Baseline ROS levels and TAC were significantly higher in patients than in controls (385 UCarr, range 225-445 vs 270, range 190-350; 280 micromolHClO/mL, range 210-351 vs 391, range 300-505 respectively; $p<0.001$, Mann-Whitney U-Test). The increase in ROS concentrations was significantly higher ($p<0.05$, Wilcoxon's test) during sepsis than FUO and bacteremia; moreover in sepsis median Nadir ROS levels (542 UCarr, range 390-704) were significantly higher ($p<0.05$) than in FUO (453 UCarr, range 342-622) and bacteremia (403 UCarr, range 390-514). When ROS was used as marker of infection or sepsis, the area under the Receiver Operator Characteristic (ROC) curve was 0.737 and 0.769, respectively. Cut-off concentration for optimum prediction in hematologic patients was ROS > 364 UCarr (Sensitivity 77.8%, Specificity 66.2%). Baseline ROS levels and TAC, significantly higher and lower respectively, in hematologic patients than in controls indicated ongoing oxidative stress probably due to the underlying disease. Moreover, at the onset of fever, the increase in oxidative stress related to infections, particularly sepsis, showed that, when an appropriate cut-off level is chosen, ROS levels can be a useful marker of infectious conditions. In conclusion, our findings have proved the usefulness of ROS concentrations and TAC when evaluating the severity of infectious condition, prompting us to extend the research to a much larger population of hematologic patients.

PO-219

FULL HAPLOTYPE-MISMATCHED HEMATOPOIETIC STEM CELL TRANSPLANTATION IN PATIENTS WITH ADVANCED HEMATOLOGICAL DISEASES

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Introduction. Bone marrow transplantation from an HLA-matched related or unrelated donor is potentially curative for patients with haematological diseases. Only 25% of patients have an HLA identical sibling. Success in a MUD search depends on HLA disparity, ranging from 60 to 70% in white individuals. Although molecular analysis achieves closer matches, it reduces the probability of finding a donor, and many patients relapse while awaiting transplantation. HLA haploidentical transplanta-

tion offers an immediate source of HSC to almost all patients. Since 1994, Perugia University researchers have reported that HSCT, in which the MHC shows a full haplotype mismatch, gives stable engraftment rates of 97% in the early stages, as well as long-term disease-free survival rates. We report our experience in our Institution. Patients and Methods. Between December 1998 and January 2005 we performed haploidentical transplantation in 12 patients (11 males and 1) of median age 39 yy (r.18-61 yy). All had advanced haematological disease (3 cases of ANLL in I CR and unfavourable risk factors, 3 cases of ANLL in II CR, 1 case of ANLL resistant, 1 case of ANLL relapsed, 1 case of blastic crisis of CML, 2 cases of ALL resistant, 1 case of lymphoma resistant) and received a conditioning regimen based on TBI single dose, Fludarabine, Thiotepa and ATG rabbit in 8 patients and Fludarabine Thiotepa, ATG rabbit and Melphalan in 4 patients. Eleven donors were NK alloreactive versus recipient for locii B and/or C of HLA. The graft consisted of peripheral blood stem cells (PBSC) that had been mobilized in the donor with G-CSF at the dosage of 10 micrograms/kg subcutaneously. T-cell depletion of PBSC was achieved by E-rosetting followed by positive selection of CD34+ cells in 3 cases and using only $CD34^+$ cell immunoselection by Isolex 300i in 9 cases. No post-transplantation graft versus host disease prophylaxis was administered. Results. The median number of CD34+, CD3+ and CD19+ cells infused was $6{,}9\times10^6$ /kg(r.3,5-10),5×10^5/kg(r.0-20) and 10×10^5/kg(r.4-30), respectively. One patient early died for hemorrhagic disease. Primary engraftment was achieved in 9 of 11 assessable patients. One of two patients who rejected the primary graft, engrafted after a second transplantation and immunosuppressive therapy. Acute GvHD developed in two cases. Overall, one patient is alive and in CR after 88 months; the others died of infection (6), graft failure (1), acute GvHD (1), hepatic complications (1) and progression disease (1).Conclusions. This procedure provides reliable, reproducible $CD34^+$cell purification, high engraftment rates and prevention of GvHD. The late immunological reconstitution, infection complications and, later, unpredictable adverse events remain the major problems for this type of transplant. However, haploidentical transplant emerges as a concrete alternative source of stem cells for patients with urgent need for transplantation, lacking a matched donor.

PO-220

NOT PUBLISHED

PO-221

ORAL VALGANCICLOVIR TREATMENT FOR CYTOMEGALOVIRUS DISEASE IN ALLOGENEIC STEM CELL TRANSPLANT RECIPIENTS

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Valgancyclovir is a valyl ester of ganciclovir which is hydrolyzed to ganciclovir before reaching the systemic circulation, having higher bioavailability than oral ganciclovir. Despite the efficacy of valganciclovir has been demonstrated in immunosuppressed patients (CMV retinitis in AIDS patients, solid organ transplant recipients, patients treated with alemtuzumab), no data exist for the established dose and the indication of this formulation as CMV pre-emptive therapy in stem cell transplantation (SCT). Starting from July 2004, valganciclovir at a dose of 900 mg os once a day, was administered in 8 out-patients submitted to allogeneic SCT as pre-emptive therapy on the basis of detection of primary or reactivated CMV infection by positive antigenemia (Ag), or positive PCR. The median age was 43 years (range 21- 54 years). They were affected by acute myeloid leukemia (4), aplastic anemia (1), acute lymphoblastic leukemia (2) and low grade non-Hodgkin lymphoma (1). Six patients obtained complete remission after transplant. Acute GVHD occurred in 7 patients and chronic GVHD was noticed in 3 patients. Immunosuppressive regimen consisted of cyclosporine and methotrexate for all patients with addiction of steroids and mycophenolate or tacrolimus based on the development of acute or chronic GVHD. Antigenemia and PCR DNA for CMV were monitored twice a week. The median time of positivization of Ag and/or PCR for CMV was 45 days after transplant (range 35-63). Among evaluable patients, the mean baseline antigenemia level was 2/200.000 cells (range 1/200.000- 5/200.000), whereas the mean level of Dna viral copies was 6×10^3/mL (range 0.320×10^3-24×10^5). The negativization of PCR and/or Ag for CMV occurred in 6/8 patients (75%) at a median of 2 weeks from starting valganciclovir (range 2-3 weeks). One patient required further CMV treatment for a 2nd re-activation, at 252nd day, but obtained a rapid negativization after 1 week of therapy. Two patients, not achieving negativization, were shifted to foscarnet but developed CMV pulmonary disease and died at 189 days and 431 days. The cause of death was not attributable to CMV because they also developed recurrence of their malignancy. No significant increased myelo or nephro-toxicity was observed. Oral Valganciclovir was well tolerated showing efficacy and safety without significant hematological and/or extra-hematological toxicity; moreover it allowed good compliance and outpatient management.

PO-222

CHIMERISM STATUS IN A DOUBLE CORD BLOOD UNIT TRANSPLANT: FROM EARLY MIXED TO FULL DONOR ENGRAFTMENT

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Background. Use of HLA-mismatched umbilical cord blood allows to transplant patients who lack related HLA-identical or matched unrelated donors with low incidence of graft versus host-disease (GVHD). An important disadvantage of UCBT is the limited number of stem cells available for transplantation, that may contribute to the delay of donor engraftment, especially in adult population. Recently, multiple-UCBT have been performed with successful engraftment in adult patients. Here we report on a patient treated with a double UCBT and sequentially monitored for chimerism at the molecular level. *Case report.* A 37-year old-man of 100 Kg b.w. with T-acute lymphoid leukemia (T-ALL) in second partial remission and without related HLA sibling donor or marrow unrelated donor (MUD) was submitted to double UCBT. Two UCB HLA-mismatched units were selected from the NetCord. They respectively were a male and a female unit and they both shared 4/6 HLA disparities between each unit and the recipient, specifically HLA-A and DRB1.The preparative regimen consisted of Fludarabine 150 mg/m^2, Tiothepa 10 mg/Kg, antithymocyte globulin 8 mg/kg, Busulphan 9.6 mg/Kg i.v. for 4d. GVHD prophylaxis consisted of CSA and PDN. Sequential molecular monitoring of chimerism status was evaluated by mini and microsatellite analysis, using PCR amplification of VNTR and STR sequences. The total dose of infused NC from both the UCB units was 2.58×10^7/Kg, CD3+cells 0.69×10^7/Kg, CFU-GM 1.5×10^4/Kg, CD34+cells 0.94×10^5/Kg. *Results and conclusion.* Complete hematological remission and neutrophil engraftment were achieved at at day +45. A mixed (triple) chimerism was documented at day +10 in bone marrow (BM) and peripheral blood (PB) where co-expression of the recipient and the two UCB polymorphic loci was documented. In particular, we observed at this time point prevalence of one of the two UCB-unit fingerprint over both the other UCB and the residual recipient haematopoiesis. At days +30 and +40, molecular analysis documented a conversion to full donor both in BM and PB. No residual recipient fingerprint was documented in any compartments, while the engraftment of both UCB donors was again documented with the persistent prevalence of the one UCB fingerprint vs the other donor counterpart. The patient developed grade II aGVHD (day+15) responding to steroid treatment. He remained in complete remission at +60 days. In the majority of multiple UCBT (about 76%) haematopoiesis is accounted for by a single donor, while the remaining patients have engraftment of both donors. In our case, preliminary evaluation in the short term indicate engraftment sustained by both donors with prevalence of the UCB unit with higher NC and CD3 dose. Further studies are warranted to better elucidate the biology of double unit UCBT and if this strategy may improve the outcome of UCBT in adults.

Myelodysplastic Syndromes and Bone Marrow Aplasia

PO-223

WT1 IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES: A USEFUL MOLECULAR MARKER FOR RISK ASSESSMENT

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Background. Myelodysplastic Syndromes (MDS) are clonal hematopoietic stem-cell disorders characterized by ineffective dysplastic hematopoiesis involving one or more cell lineages and characterized by peripheral-blood cytopenias and a high risk of progression to acute myeloid leukemia (AML). According to WHO classification, MDS can be classified in these following groups: refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess of blasts type I and II (RAEB I and II), refractory cytopenia with multilineage dysplasia (RC+Dys), del (5q) sindrome, and MDS unclassifiable (MDS unclass). The Wilms' tumor gene (WT1) is a tumor suppressor gene coding for a zinc-finger transcription factor located on chromosome 11p13, which was originally identified for its involvement in the pathogenesis of the Wilms' tumor. In normal peripheral blood (PB) and bone marrow (BM), WT1 expression is reported to be low and sometimes undetectable even by RT-PCR. By contrast, WT1 is highly expressed in most acute leukemias, and its level of expression is associated with the presence, persistence, or reappearance of leukemic hematopoiesis. *Aims.* WT1 gene expression could represent a useful marker in MDS to establish prognosis and progression of disease. *Methods.* BM samples from 36 MDS patients (16 RA, 7 RAEB I, 4 RAEB II, 4 RARS, 3 deletion of 5q, 2 MDS unclass) were tested for WT1 expression at diagnosis and after 6 months. WT1 gene expression was evaluated by methods of real-time quantitative PCR (RQ-PCR). *Results*. At diagnosis, 21BM samples (10 RA, 6 RAEB I, 4 RAEB II, 1 RARS, 1 MDS unclass) expressed WT1 transcript amounts greater than the ranges level. The degree of WT1 expression was highly correlated with the type of MDS, was much higher in RAEB I and II compared with RA, and other types, and increased during disease progression. Moreover, a significant correlation was found between WT1 expression levels, blast cell percentage, and the presence of cytogenetic abnormalities. The patients received only a supportive therapy if necessary. After 6 months, 9 patients (2 RA, 5 RAEB I, 2 RAEB II) converted to AML. All of these patients showed at diagnosis an high WT1 expression level and a further elevation of WT1 expression after 6 months. *Conclusion*. WT1 expression has been previously reported to be increased also in myelodysplastic syndromes. In this study, the data obtained show that in most MDS, including a large percentage of RA and almost the total number of RAEB I and II, WT1 is expressed above the range observed in normal controls in BM and that its expression is directly correlated with the type of MDS. In addition, even within each subgroup, a strong association is present between the level of WT1 expression and the blast percentage and the presence of cytogenetic alterations. The identification of a molecular marker so able to establish the tendency of MDS to progression can be of great help in decision making for MDS patients. In conclusion we believe that WT1 can be introduced as a additional marker to the standard parameters already considered in risk assessment for MDS.

PO-224

GLYCOSYL-PHOSPHATIDYL-INOSITOL (GPI)-DEFECTIVE GRANULOCYTES FROM PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA (PNH) PATIENTS SHOW INCREASED BACTERIAL INGESTION BUT REDUCED RESPIRATORY BURST INDUCTION

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Paroxysmal Nocturnal Haemoglobinuria (PNH) is characterized by the emergence of a GPI-defective clonal haematopoieis. Its clinical features are hemolytic anemia, cytopenia and thrombosis. Circulating monocytes and granulocytes are largely GPI-defective in PNH patients. This study aims to investigate the granulocyte functional properties in PNH. We analyzed bacterial-dependent intracellular ingestion and the consequent activation of oxidative burst in GPI-defective granulocytes from four neutropenic PNH patients. Our data show a significant increase in the ability of GPI-defective granulocytes to ingest opsonized bacteria. In addition, an impaired respiratory burst effectiveness in response to two independent bacterial stimuli the N-formyl-MetLeuPhe (fMLP) synthetic bacterial peptide and E.Coli, was revealed. The occurrence of neutropenia and the severe impairment of oxidative burst, occurring in Chronic Granulomatosis Disease (CGD), were unable to significantly affect phagocytosis. The reduced burst effectiveness of GPI-defective granulocytes was maintained after treatment with phorbol 12-myristate 13-acetate (PMA), a pharmacological stimulus able to extensively recruit and to trigger intracellular Protein Kinase C (PKC). Moreover, blocking of PKC has been observed to severely affects granulocyte respiratory burst with a mild effect on the phagocytosis. These data suggest that a modulation of intracellular PKC levels could be involved in the pathogenesis of the impaired granulocyte oxidative burst observed in PNH granulocytes. In this context, the lack of the GPI-linked molecules (CD55 and CD59) on cellular membrane produces increased level of activated complement fractions in PNH. The consequent excess of activated C3 molecules could act as continuous stimulus mediating increased ingestion effectiveness and consequent depletion of intracellular PKC levels. This effect could be involved in the pathogenesis of the observed impaired oxidative burst generation in GPI-defective granulocytes.

PO-225

REAL-TIME PCR AS A TOOL FOR QUANTITATIVE ANALYSIS OF PI-PLC-β-1 GENE EXPRESSION IN MYELODYSPLASTIC SYNDROMES

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Phosphoinositide-specific phospholipase C (PI-PLC) β-1 is a key enzyme in nuclear signal transduction, and it is involved in many cellular processes, such as proliferation and differentiation. In particular, the involvement of the PI-PLC-β-1 gene in erythroid differentiation lead us to investigate this gene in patients affected by high-risk myelodysplastic syndromes (MDS). By using fluorescence in situ hybridization (FISH) analysis, we have previously evidenced that, in MDS patients with normal GTG banding and a fatal outcome, the PI-PLC-β-1 gene undergoes a monoallelic and interstitial deletion. Real-Time PCR is characterized by high sensitivity, excellent precision and large dynamic range, and has become the method of choice for quantitative gene expression measurements. In the present study, we have performed a relative quantification real-time polymerase chain reaction (PCR) analysis on all of the MDS patients tested for FISH analysis. Furthermore, we have evaluated the expression of the PI-PLC-β-1 gene on healthy donors and the HL60 cell line, which is useful for testing the accuracy of the technology because of its low expression of PI-PLC-β-1. To analyze and quantify the levels of the two different splicing variants of PI-PLC-β-1 gene (1a and 1b), we have used a TaqMan isoform specific probe. We have seen that all of the MDS patients have higher levels of the PI-PLC-β-1 mRNA compared to the HL60 cell line as expected, but lower levels compared to the healthy donors. Furthermore, MDS blasts always express higher levels of PI-PLC-β-1b mRNA compared to PI-PLC-β-1a mRNA. Our data support the contention that the deletion of PI-PLC-β-1 gene is indeed responsible for a reduced expression of the enzyme. In addition, the splicing isoform 1b, which is only nuclear, seems to be somehow partially preserved compared to the 1a isoform which is nuclear and cytoplasmatic, hinting at a possible imbalance of the nuclear versus cytoplasmatic PI-PLC signaling which, in turn, could affect the cell cycle progression of MDS blasts.

PO-226

A PILOT STUDY OF BORTEZOMIB (VELCADE) FOR THE TREATMENT OF MYELODYSPLASTIC SYNDROMES

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Bortezomib is a proteasome inhibitor which has been demonstrated to be effective in some haematological malignancies, mainly multiple myeloma and mantle cell lymphoma. Of interest, inhibition of the proteasome complex could provide an anti-TNF effect via suppression of the NF-kB pathway, thus possibly inducing a reduction of the intramedullary apoptotic cell death which characterizes the ineffective hematopoiesis of myelodysplastic syndromes (MDS). To date, however, no data have been extensively published about the possible therapeutic role of bortezomib in MDS. Aiming to contribute to this issue, we treated with bortezomib (1.3 mg/sqm days 1, 4, 8, 11, with an interval of ten days, every three weeks), seven patients affected by MDS (five were males and two females, aged 61 to 81). According to WHO classification, there were 2 refractory anemias (RA), 1 refractory anemia with ring sideroblasts (RARS), 1 refractory anemia with blast excess type 2 (RAEB-2), 3 refractory cytopenias with multilineage dysplasia (RCMD). All patients had normal karyotype and were transfusion dependent (Hb levels less than 8 g/dl). Prior therapies had included recombinant erythropoietin or darbepoetin (+/- the adjunct of G-CSF), thalidomide, steroids, androgens and vitamins, without or with loss of efficacy. Two patients (1 RA, 1 RAEB-2) received only one complete cycle (four administrations) due to the appearance of atrial fibrillation and intestinal hemorrhage, respectively. Both these events, however, had been also previously observed during the clinical history of patients, before the treatment with bortezomib. Five patients (1 RA, 1 RARS, 3 RCMD) received three complete cycles (12 administrations each). Minimal dose reductions were needed in three patients, due to a decrease in platelet count, never higher, however, than 50% of initial values and in absence of other relevant side effects. According to recently published (Blood, 2006), modified IWG criteria, a single hematological improvement (erythroid response: Hb increased by 1.7 g/dL) was observed in a patient with RCMD. The response was lost after the voluntary interruption of the treatment (three cycles received in total). Hemoglobin and white blood cell counts remained substantially unchanged during and after the treatment with bortezomib, without any modification of transfusional needings in other treated patients. Marrow morphology did not change, even in the responding patient. These preliminary data suggest that bortezomib provides limited benefits, in terms of erythroid improvement, in transfusion-dependent patients with MDS.

PO-227

THE COMPLEMENT INHIBITOR ECULIZUMAB TO TREAT PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PATIENTS: A SINGLE CENTER EXPERIENCE WITHIN THE TRIUMPH, SHEPHERD AND EXTENSION STUDIES

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Paroxysmal nocturnal hemoglobinuria (PNH) is a hematological disorder characterized by the clonal expansion of one or a few hematopoietic stem cells which are incapable of glycosylphosphatidylinositol (GPI)-anchor biosynthesis, due to an acquired somatic mutation in the phosphatidylinositol glycan class A (PIG-A) gene. Affected progeny cells lack from their surface all GPI-anchored proteins, including the complement regulators CD55 and CD59; thus, PNH red blood cells (RBCs) inefficiently prevent both complement activation and membrane attack complex formation, resulting in chronic intravascular hemolysis, hemoglobinuria and anemia. Other cardinal symptoms of PNH are marrow failure and propensity to venous thrombosis; their relationship with the genetic defect are still to be elucidated; in the latter case a platelet activation due to inefficient complement blockade has been postulated. Eculizumab is a humanized monoclonal antibody against the complement fraction 5, which inhibits the terminal membrane attack complex formation; in a pilot study, eculizumab demonstrated clinical efficacy in a small cohort of PNH patients. Three different international phase III multi-center GCP-conducted studies are ongoing to evaluate efficacy and safety of eculizumab in PNH patients. The TRIUMPH trial is a randomized, double-blind, placebo-controlled study, enrolling patients transfusion-dependent (>3 units per year), non cytopenic and with a PNH RBC clone >10%; safety and efficacy in the two arms are the primary endpoint. The Shepherd trial is an open-label study looking to safety issues, and include also patients with lower transfusional requirement or mild cytopenias. The Extension study is an open-label study designed as treatment extension of the two previous trials to investigate long-term safety, and is open to patients completing the TRIUMPH or the Shepherd study only. Here we report our experience within these international studies; we evaluated 20 PNH patients followed by or referred to our institution. Three patients were enrolled in the TRIUMPH study, and 5 in the Shepherd study; this represents the 5% of the worldwide enrollment goal (which was 75 and 85 patients in the two studies, respectively). After the screening/observational phase, all the patients entered the treatment phase, which included an induction followed by a maintenance period. Patients received either eculizumab 600 mg/week for 4 weeks and then 900 mg every two weeks; in the TRIUMPH study a placebo arm, placebo with the same administration schedule, was included. All the 8 patients did not experience any relevant adverse events, not even mild infusion reactions; the 3 patients enrolled in the TRIUMPH study completed the study, and were subsequently enrolled in the Extension study, where they received eculizumab regardless the previous treatment. Other 5 patients are currently on eculizumab within the Shepherd trial, and are planned to start the Extension study within a few weeks. Eculizumab is an attractive treatment for the control of intravascular hemolysis in PNH patients, a condition still lacking effective therapies. Results from the ongoing international trials are prompt to be published, and will tell us whether this product may be beneficial to stabilize hemoglobin and possibly improve associated morbidities in PNH patients, as well as its safety profile.

PO-228

POSSIBLE MECHANISMS OF ACTION OF THALIDOMIDE IN MYELODYSPLASTIC SYNDROMES

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Although thalidomide has been demonstrated to be effective in improving anemia of patients with low-intermediate-risk myelodysplastic syndromes (MDS) in about 20-30% of treated subjects, the exact mechanism(s) by which the drug acts are still not elucidated. We investigated the possible effects of thalidomide in modifying angiogenesis, apoptosis, adhesion capacities and immunological response in 65 patients, diagnosed according to WHO criteria, with low-intermediate1-risk MDS (IPSS score), we treated during the last 6 years. Eighteen of these patients (27.6%) showed a hematologic improvement (erythroid response), according to recently published (Blood, 2006), revised IWG criteria. In particular, we observed that: a) Thalidomide stimulated some physiological mechanisms of compensation to correct anemia, including production of endogenous erythropoietin and fetal hemoglobin. b) With respect to angiogenesis, which is generally increased in bone marrow of MDS patients, a paradox effect was seen: in fact, thalidomide often determined an increase (rather than a reduction, as expected by an anti-angiogenesis agent), in marrow microvessel density (immuno-histochemical assay) and plasma levels of some angiogenic cytokines, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF), and hepatocytic growth factor (HGF), although with some relevant differences among single patients. As for previous observations, we interpreted these findings as a possible, further mechanism inducing an improvement of marrow oxigenation in anemic patients. c) Thalidomide reduced apoptosis, the major cause of disturbance in production of effective myelopoiesis in MDS. This was suggested by decreased levels of circulating pro-apoptotic cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 α (IL-1 α), by reduction of circulating levels of soluble transferrin receptor (a surrogate marker of total

erythropoiesis and, therefore, of ineffective erythropoiesis in MDS), as well as by immuno-histochemical (annexin V) Tunel technique. d) Thalidomide induced a significant reduction of peripheral blood CD4+ cells and an increase of lymphocytes with NK and γ-δ phenotype, without significantly affecting CD8+ lymphocyte subsets. e) Thalidomide had no effects on circulating adhesion molecules, such as V-CAM-1 and E-selectin serum levels. Overall, however, marrow and peripheral blood modifications induced by thalidomide were heterogeneous, sometimes contradictory and variously combined in single subjects, often independently upon their clinical response. Such a complexity of effects likely reflects and further confirms the biological differences and peculiarities which characterize patients with MDS.

PO-229

BIOLOGICAL AND CLINICAL RELEVANCE OF MATRIX METALLOPROTEINASES 2 AND 9 IN MYELODYSPLASTIC SYNDROME

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Matrix metalloproteinases (MMP) are a family of zinc-dependent endopeptidases which are able to degrade all the protein components of the extracellular matrix. MMP-2 (type IV collagenase, gelatinase A) and MMP-9 (type V collagenase, gelatinase B) have been implicated in tumor progression and metastasis and, recently, it was suggested that these enzymes may also contribute to leukemic dissemination. We analyzed the expression of MMP-2 and MMP-9 in bone marrow cells from 129 patients with myelodysplastic syndrome (MDS) (49 RA, 26 RARS, 32 RAEB, 3 RAEB-t, 19 CMML), not previously treated, and from 45 non hemopathic subjects, in order to evaluate whether abnormalities in their expression were associated with relevant laboratory and clinical findings. Moreover, a possible correlation was investigated between MMP positivity and altered apoptosis level, as measured by TUNEL technique, or altered proliferation, as evaluated by MIB-1 immunostaining. MMP-2 and MMP-9 were detected on bone marrow smears by an immunoalkaline phosphatase method (streptavidin-biotin complex) using primary murine monoclonal antibodies raised against human MMP-2 (clone A-Gel VC2, NeoMarkers) and human MMP-9 (clone IIA5, NeoMarkers). In normal samples MMP-2 was detected only in occasional myeloid cells, whereas MMP-9 was expressed in some 20-30% of maturing myeloid cells. In MDS the percentages of cells positive for MMP-2 (median 36%, IQR 22-47%) and MMP-9 (median 40%, IQR 28-53%) were significantly higher than those observed in normal controls (p=0.0000 and 0.04 respectively). There was a close relationship between MMP-2 and MMP-9 expression in MDS myeloid cells (p=0.003) and also many erythroblasts expressed both enzymes. In early MDS (RA and RARS) percentages of MMP-2 and MMP-9 positive cells higher than in advanced forms were observed (p=0.01 and 0.001 respectively). In advanced MDS a tendential inverse correlation between MMP-2 and TUNEL positivity was identified by the Spearman correlation test (p=0.06); in the whole MDS group MMP-9 as well as MMP-2 expression was independent of the proliferative rate. A significant inverse correlation between either MMP-2 or MMP-9 and bone marrow blast cell percentage was observed (p=0.03 and 0.01 respectively), but no significant relationship was found between MMP levels and clinical and laboratory features such as age, leukocyte count or karyotype. A low MMP expression was associated with significantly shorter overall survival. Among early MDS patients treated with thalidomide the cellular expression of MMP-2 and MMP-9 as well as apoptosis decreased in bone marrow erythroblasts of responsive cases. In conclusion, for the first time the relation between MMP abnormal expression profile and other biological and clinical features has been evaluated systematically in MDS. Our findings suggest that the production and release of these enzymes may influence hematopoietic cell death and behaviour, possibly by the processing of regulatory proteins in marrow, with a potential prognostic significance for disease progression. On the other hand, MMPs may represent specific targets for therapeutic intervention.

PUBLISHED ONLY

PUB-01

OPTIMIZATION OF SIMPLE PCR ASSAY FOR DETECTION OF GLOBIN GENE DELETIONS IN A THALASSEMIA PATIENTS

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Thalssemia is the most common genetic disorder in the world. Pakistan has the highest incidence of α and β thalassemia in the world. Mutations in globin gene have been attributed to thalassemia. In case of α thalassemia, deletions in globib gene are responsible in 90% of the patients. We developed a very simple PCR assay to detect common deletions in α thalassemia patients in our populations. Specific primers were used to amplify genomic regions responsible for globin gene deletions corresping to α thalaasemia. PCR products were run on agarose gel to detect deletions directly. Deletions α 3.7 and α 4.2 were detected in patients. Our study reveals that like other populations, deletions in globin gene are related with α thalassemia in Pakistani patients. Moreover, our optimized PCR protocol is very simple, cost effective method to screen globin gene deletion mutants in α thalassemia patients.

PUB-02

POLYCYTHEMIA VERA AND BLOOD-LETTING THERAPY: OUR EXPERIENCE

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Blood-letting therapy is a medical treatment useful in two kind of diseases: erythrocytosis and hemochromatosis. In the first group of pathologies, an increased hematocrit (Hct), with a consequent hyper-viscosity syndrome, is the main clinical feature; while in the second one, excessive iron absorption and deposit determine fibrosis of organs and tissues, with a secondary functional insufficiency. In this study, we analyze the therapeutic results achieved in our Departments in the last 3 year. We have studied 69 patients, 45 men (with an age of 56.6±15.4 years, mean ± Standard Deviation) and 24 women (58.3±16.1 years), affected with Polycythemia Vera (PV), diagnosed according to Polycythemia Vera Study Group's criteria, treated only by bloodletting. Therapeutic targets were to achieve a Hct < 45% in men and < 42% in women. A variable number of phlebotomies has been performed according to initial Hct of each patient. In particular, male patients received 4.1±1.6 venesections, while in females 3.2 ± 1.5. Each weekly phlebotomy amounted to 400 ± 50 mL of whole blood. No side effects, adverse reactions or complications were registered. Retrospective analysis of Hct and Haemoglobin (Hb), assayed before and after each venesection, showed reduction of Hb = 1.08±0.33 g/dL and of Hct = 2.92±0.64%. Of course all patients resulted iron depleted (Ferritin < 50 ng/mL and Transferrin Saturation < 20%) and an iron-limited hematopoiesis was obtained in all cases. Blood-letting therapy is an economic and effective medical treatment without complications for patients. Contraindications are only anemia or hypoproteinemia, while age and associated pathologies not represent limitations to the treatment. Iron-depletion must no limit the treatment too; on the contrary it represents an additional target of therapy, because it lets the martial deposit and determines an iron-limited hematopoiesis. This last one allows us to maintain for a long time the achieved therapeutic results. In conclusion phlebotomy remains the main treatment of PV, whereas myelosuppressive agents may augment the benefit of using phlebotomy for thrombosis prevention in high-risk patients.

PUB-03

THE ROLE OF ERYTHROCYTAPHERESIS IN HEMATOLOGICAL DISEASES

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An alternative therapy to phlebotomy for various hematological diseases, such as polycythemia vera (PV) and secondary erythrocytosis, is therapeutic erythrocytapheresis (EA) performed by cell separator. This technique is useful in order to quickly remove a large volume of red blood cells (RBC), saving plasma proteins and clotting factors. In fact long-term survival of these patients is essentially determined by the capacity in reduce the risk of thromboembolic complications. This last is due to the altered blood rheology of these conditions resulting by the high RBC mass. Aphaeresis is often combined with drug therapy to obtain more benefits. In polycythemia, RBC depletion to normal hematocrit (Hct) values is the first line therapy and it should be preferred to chemotherapy because of the long-term risk of acute leukemia or other secondary malignancies. RBC depletion is accomplished much more effectively and rapidly by EA than by repeated phlebotomies and is generally well tolerated and accepted by the patients. Nonetheless for aphaeresis, there is no standard protocol and clinical experience with this therapy remains highly anecdotal. The main indication for EA in these patients is high risk Hct >55-60%; in fact this value may be reduced to the normal range by only 1-2 apheretic procedures. The long-lasting effect is also due to the massive loss of iron, an essential factor for erythropoesis; in fact, after the first treatment cycle, the median observed interval between 2 EA, when Hct is returned in normal range, is about 6 months. In order to evaluate the efficiency and safety of this technique in these pathologies we reported a retrospective analysis of our experience with 20 patients affected by PV and 36 by secondary erythrocytosis: from 2002 to 2006 we performed 169 procedures using Haemonetics MCS plus. Before every aphaeresis procedure we verified Hb (in median 18.7 g/dL), Hct (in median 58.2%), viscometry, coagulation test, EGA, PFR and ECG. 8 patients were treated with 1 EA, 12 with 2, 15 with 3, 13 with 4 and 8 with 5. After the whole cycle of apheresis, the total mean volume of concentrated RBC removed was 576 mL (range 426-830), Hb post was 14.3 g/dL and Hct post was 42.4%; hematic viscosity was significantly reduced, while tissue oxygen tension increased: the improvement of symptomatology and hematochemical parameters was maintained on the average for 6 months. All the procedures were well tolerated and light side effects (paresthesias citrate-depending in 18 aphaeresis) were easily controlled. Erythrocytapheresis, compared to phlebotomy, has the advantage of selectively removing RBC without loss of clotting factors, platelets and plasma proteins. Although it has relatively high costs, we noticed a decrease of hospital recurrence (about 50-65%) in patients treated with aphaeresis.

PUB-04

MAINTENACE THERAPY WITH MONOCLONAL ANTI-CD20 (RITUXIMAB) IN PATIENTS AFFFECTED BY HIGH OR LOW GRADE NON HODGKIN LYMPHOMA: A SINGLE CENTER EXPERIENCE

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When associated to chemotherapy in front-line treatment monoclonal anti-CD20 (Rituximab) has improved complete remission rates in Non Hodgkin Lymphoma. In order to improve progression free survival it has been proposed as single agent after complete remission has reached. From 2003 a program of maintenance with Rituximab after chemotherapy in newly diagnosed Non Hodkin Lymphoma patients and in patients who obtained a second remission after relapse was carried out in our institution. A total of 12 patients were treated with rituximab as maintenance therapy at the dodage of 375 mg/m^2 once every two months. Main characteristic of patients were as follows: 10 were affected by newly diagnosed LNH; 2 were follicular lymphomas, 4 were diffuse large B cell lymphomas and 4 marginal B cell lymphomas. 2 patients were treated after second remission and both were affected by follicular lymphoma. Mean age of patients was 61,66 years (range 50-85). Stage of the disease was III-IV for all patients. 8 patients presented with comorbidities (3 ischemic cardiopathy, 3 diabetes and 2 chronic bronchitis). Up front therapy was R-CHOP 6 cycles every 21 days for all 4 patients affected by diffuse large B cell lymphoma and for the 2 patients in relapse. The remaining 6 patients were treated with 6 cycles of R-FluCy. All patients reached a complete remission of the disease confirmed by TC scan, PET and marrow biopsy and a month after the last cycle of chemotherapy all received Rituximab 375 mg/m^2 one dose every two months for a planned period of two years. Median follow-up is actually 10,66 months (range 3-24) with a patient who completed the 2 years maintenance treatment. Maintenance treatment has been well tolerated. No serious adverse events have been recorded, only 1 patient affected by follicular lymphoma and treated with fludarabine and cyclophsphamide developed autoimmune hemolitic anemia that need corticosteroid therapy. Another patient, affected by follicular lymphoma too, developed ipoγglobulinemia. All patient are in complete continous remission without relapsing disease.Larger series and longer follow-up is needed to confirm that maintenance approach is safety and efficacy in improving progression free survival and overall survival in patients affected by high or low grade Non Hodgkin Lymphomas.

PUB-05

SERUM LEVELS OF INTERLEUKIN-16 AS AN IMPORTANT PROGNOSTIC FACTOR IN MULTIPLE MYELOMA PATIENT WITH CUTANEOUS INVOLVEMENT

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Cutaneous plasmacytoma may occur either as a primary manifestation or as cutaneous involvement of multiple myeloma without extension from a bony lesion, both of which are uncommon. It is often associated with diffuse, systemic, late-stage disease and reflects high tumor burden, and patients with multiple myeloma have a very short survival period once specific skin lesions appear. The mechanism of cutaneous involvement is assumed to be a change in adhesion molecules able to cause changes in migration and homing. We present a case of a patient, a 79 years-old female, affected by multiple myeloma who presented skin lesions consisted of multiple nodules with erythematosus or violaceous coloration in the abdominal's region, in the arms and in the thighs. Patient showed an increasing of serum levels of Interleukin-16 (344 pg/mL) as compared with normal controls (172.28±71.95) and with a group of myeloma patients at the same stage of disease (188.05±96). She also showed an increase of other negative prognostic factors (β2microglobulin, CRP) and poor response to treatment. It was reported that serum IL-16 levels are higher in myeloma multiple patients and that there is a strong correlation between the serum IL-16 level and disease activity. The significance of the changes in IL-16 levels in patient with cutaneous involvement could be important in MM growth and dissemination. IL-16 is in fact able to stimulate the production of other cytokines, such as IL-6, the more important growth factor for neoplastic plasma cells. Moreover, IL-16 induces chemotaxis of CD4$^+$ T cells contributing to their recruitment and activation, and their subsequent apoptosis, and previous reports have shown that blood levels of immune cells predict survival in myeloma patients, and subjects who have higher baseline levels of blood CD4$^+$ cells have longer survival. Further investigation should be undertaken to better determine the pathophysiologic role and the prognostic value of IL-16 in dissemined cutaneous myeloma.

PUB-06

UNUSUAL EXTRAMEDULLARY MICROMOLECULAR MULTIPLE MYELOMA: CLINICAL AND RADIOLOGICAL RESPONSE TO BORTEZOMIB +THALIDOMIDE+DEXAMETHASONE

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Micromolecular Multiple Myeloma (MM) is a rare disease with an aggressive outcome characterised by multi-organ failure and extramedullary localisation. Bortezomib, a proteasome inhibitor, exerts its activity mainly through binding a catalytic site of 26S proteasome with a consequent NFkB degradation, reduction of antiapoptotic proteins synthesis and MM cell death. In the clinical trials Bortezomib, as a single agent, showed a substantial activity in heavily pre-treated patients

who are in progressive/relapsed disease. Encouraging preliminary results, suggest an increased efficacy when Bortezomib is administered in association with dexametasone, thalidomide, melphalan or daunorubicine. In particular the association Bortezomid + dexamethasone seems to be associated with an higher response rate(SUMMIT/ CREST trials). We report on two patients, a male and a female- aged 60 and 61 yearswith at diagnosis a lambda and kappa light chains micro-molecular MM stage III A, respectively. After first line therapy (four cycles VAD) partial response was achieved in both the cases. The patients, then, underwent high dose cyclophosphamide+melphalan followed by with autologous stem-cell rescue, obtaining a complete remission. Both patients showed an MM disease relapse 39 and 13 months later, respectively; at which time bone marrow biopsy and urine immune-electrophoresis were negative. The main clinical features of relapse for the male were left eye-diplopia and left palpebral ptosis; while for the female there was right eye-esophtalmus and right clavicular swelling. Nuclear magnetic resonance (NMR) evidenced in the male the precence of an round tissue spread from the clivus bilaterally to the sphenoidal sinus; in the female the tissue spread from the frontal parietal bone to the sphenoidal maxillary sinus. Furthermore the female had a fracture of the right sternclavicula joint, which a biopsy revealed as atypical plasma cell mass. The patients underwent bortezomib+thalidomide+dexametasone therapy. Bortezomib and dexamethasone were administered at dosages of 1.3 mg/sqm and 40 mg respectively, every three weeks on days 1-4-8-11. Thalidomide dosage was progressively increased to 150 mg daily 1 month later. This therapy promptly induced diplopia resolution in the male and pain relief in the woman. CT and NMR, after second cycle, showed a complete disease regression in both patients. To date the patients are in continuous complete remission for 3,5 and 5 months, they are being given the fifth and the seventh cycle, respectively. It is to be emphasized that in the female a complete sclerotic bone regeneration of the above mentioned fracture took place. Our experience has proved that this combined regimen is a very efficacious and safe approach in micromolecular MM. The distinguishing features of the reported patients were their unusual disease localization and the positive response that they demonstrated to the application of this combined treatment. These cases thus highlight the efficacy of bortezomib + thalidomide + dexametasone therapy on extramedullary localisation in patients with advanced MM. As recently reported the anti myeloma effect of Bortezomib, as a single agent, seems to be associated with a significant improvement of osteoblastic function, thus the bone anabolism may hamper MM growth(Zangari et Al, Heider *et al.* 2005). These observations may explain the early bone regeneration observed in our female. Available data has not yet established the best combined therapy, and only a long term follow up will define whether this complete response will translate into a prolonged progression- free survival. Our experience has shown that thalidomide combined with dexametasone and bortezomib may have an enhanced therapeutic effect in extramedullary disease. Further studies on a larger cohort of patients with prolonged follow up are also needed to define the role of this combined schedule a maintenance therapy in these high risk patients.

PUB-07

COMBINATION THERAPY PAD (PS-341/BORTEZOMIB, DOXORUBICIN AND DEXAMETHASONE) IN TWO CASES: PLASMACELL LEUKEMIA AND PLASMOCYTOMA OF THE RIB CAGE

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Background. Proteasome inhibitors are very important drugs in the therapy of Multiple Mieloma (MM). Among these, Bortezomib has shown to be particularly effective in patients with relapsed or refractory MM. It has a greater efficacy when associated to dexamethasone. Synergic effect with doxorubicin has been demonstrated. The association of Bortezomib, Doxorubicin and Dexamethasone (PAD) is therefore rationale. Each cycle lasts 21 days. Bortezomib is administered intravenously at the dosage of 1.3 mg/m^2 at days 1,4,8,11. Oral or intravenous Dexamethasone is administered at the dosage of 40mg/m^2 at days 1-4, 8-11, 15-18 (first and third cycle) and at days 1-4 (second and fourth cycle). Intravenous Doxorubicin is administered at the dosage of 9mg/m^2 at days 1-4 of each cycle. We used this regimen in two patients: one had plasmacell leukaemia, the other one a plasmocytoma of the chest wall. *Case 1.* A woman aged 73 was referred to our clinic with a diagnosis of right hemothorax, multiple rib fractures and suspected chronic lymphoproliferative syndrome. Her clinical conditions were poor, she had productive cough, dyspnoea, and back and costal pain. Thorax X-Ray showed bilateral pleural effusion. At cardiac U.S. there was the presence of pericardic effusion, with a E.F. of 59%. MRI of the spine and scheletal X-Ray were not performed because of insufficient cooperation of the patient. Blood cell count showed macrocytic anemia (Hb 11.2g/dL, MCV 105 fl) and lymphocytosis (WBC 34.000/μL, L: 84%). Platelet count, clotting tests, renal function, calcemia, RCP, ESR and β 2 microglobulin were normal. She had hypoprotidemia (5.2 g/dL) with normal albumin (3.6 g/dL). Serum protein electrophoresis showed hypoγglobulinemia (γ globulins: 4.6%). Serum and urine immunofixation did not show any monoclonal protein. Bone marrow trephine and cytology on pleural effusion evidenced a diffuse lymphoplasmacellular infiltrate. Immunophenotyping of peripheral blood showed CD38/CD56 positive cells. We concluded for a diagnosis of plasmacell leukaemia. The patient received 4 cycles of PAD therapy from September 2005 to January 2006. Chemotherapy was well tolerated without significant side effects. At present the patient is in haematological remission (Hb 11.8 g/dL, MCV 90.5fl, WBC 4.600/microlitre (N: 74% L: 20%, M:6%), PLT 173.000/μL. Hypoprotidemia still persists, but there is a mild increase of the γ globulins fraction (8.4%). Unfortunately she has relapsing pleural effusions requiring thoracentesis. *Case 2.* A man aged 81 was admitted to our clinic because of cough, dyspnoea and a mass on the back wall of the chest. Thorax X-Ray showed a mass destroying the rear arch of the fourth rib. At CT scan the dimension of the mass was 5×10 cm. The histological diagnosis was low grade plasmocytoma. Blood cell count, calcemia, renal function, were normal. ESR, RCP, β 2 microglobulin were increased. He had hyperγglobulinemia (γ globulins: 36.2%), increased IgG levels (38.3g/L) and a seric monoclonal protein IgGk. He was HCV positive without impairment of liver function. Scheletal X-Ray confirmed the erosion of the fourth rib and showed the involvement of the second and fifth ones. Bone marrow was not infiltrated. We diagnosed low grade plasmocytoma of the rib cage The patient was treated according to PAD regimen (four cycles from September 2005 to January 2006). During therapy he mainly complained of leg pain. At restaging blood cell count, bone marrow, ESR, RCP, β 2 microglobulin serum protein electrophoresis were normal. At CT scan of thorax there was no evidence of any mass. The patient is alive. *Conclusions*. Despite the short follow up, we can affirm that using PAD chemotherapy the patient with low grade plasmocytoma obtained a very good response, while the one with plasma cell leukaemia obtained a haematological remission and an improvement of clinical conditions, despite the relapsing pleural effusion.

PUB-08

SEVERE PANCYTOPENIA SECONDARY TO BORTEZOMIB: A CLINIC CASE

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Bortezomib (Velcade®) is a novel proteasome inhibitor that shown an impressive activity in relapse/refractory myeloma patients. Its antimyeloma effect exerts blocking the activation of transcription factors such as NF-kappaB and inducing myeloma cells apoptosis via caspasi 8 e 9. Major adverse effects of Bortezomib (B) are trombocytopenia (40%), peripheral neuropathy (up to 50%), anemia (30%), fatigue (50%), rash (20%), neutropenia (20%), fever (34%), infection (20%), hypotension (12%), nausea and vomiting (60%). We report a case of 58 year-old caucasian man who presented relapsed multiple myeloma (IgAkappaappa, IIIA). In front line treatment, he received two courses of VAD + 2 VAC + HD-CTX (7 gr/sm) + tandem autologous peripheral stem cell transplant (MEL 200 mg/sm conditioning regimen) followed by IFN and Zolendronic Acid as maintenance therapy for 1 year. At disease relapse, Thalidomide 200 mg/day was administrated, but it was stopped after seven months for progression disease. We planned five courses of B alone, 1.3 mg/sm iv on days 1,4,8,11 q3w. Complete response (negative immunofixation, IF), according EBMT criteria, was reached after four courses. Toxicities comprised nausea, mild fatigue, hypotension and trombocytopenia. After 5th B, an uncommon pancytopenia arised and laboratory findings shown: WBC 1,160 (Neu 33%, Lym 64%, Eos 2%, Bas 1%), HB 8.1, Ht 26, MCV 96, PLTs 19.000, LDH 176, PCR 1.2, β2M 1.5, Total Prot. 6.7, Alb 61.5, α1 4.3, α2 9.5, β 10.5, γ 14.2, IgG 10.24, IgM 0.47, IgA 1.11, Ca 8.6, BUN 24, Creat 0.7, serum and urine IF negative, serum and urine free light chains and kappa/lambda ratio normal, folate and Vit. B12 normal, IAT

and DAT both negative. Bone marrow biopsy shown marked hypo-cellularity, megakaryocyte numbers reduced, myeloid and erythroid hypoplasia without dysplastic features, plasma cells virtually absent. After three weeks, in wich patient was supported with blood transfusions and G-CSF 5 micro-grams/Kg/day, pancytopenia self limited spontaneously. Subsequently, patient underwent peripheral stem cell collection following HD Ara-C (4 gr/sm/day IVCI for three days) plus G-CSF. CD34+ stem cell collection was 2.7×10^6/Kg and 3th autotrasplant is in planning. *Conclusions*. isolated trombocytopenia, anemia or neutropenia are described as indesiderable effects of B. Otherwise, trombo-cytopenia B correlated is not associated with reduction of megakaryocytes in bone marrow, but it depends by lack platelets fragmentation and release. In our case, a reversible, self limited pancytopenia due to B, in a CR patient, was associated to severe trilineage bone marrow hypoplasia and, probably, to an important microenvironment overthrow.

PUB-09

RARE LOCATION OF SOLITARY PLASMACYTOMA

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The most common neurologic complication of Multiple Myeloma are spinal cord compression due to epidural plasmacytomas or vertebral fractures and peripheral polyneuropathy due to the presence of antibodies directed against myelin structures or due to amyloid deposits. Intracranial locations are extremely rare. Solitary plasmacytomas rarely develop in the skull, meninges, or brain. Ophthalmic signs as the initial manifestations of solitary intracranial plasmacytoma have rarely described. We report a 68-year-old man presented with optical neuropathy; computed tomography (CT) scan (Figure 1, Figure 2) showed one rare location that involves all the clivus, infiltrating the sphenoid sinus and the cavernous sinuses as well.

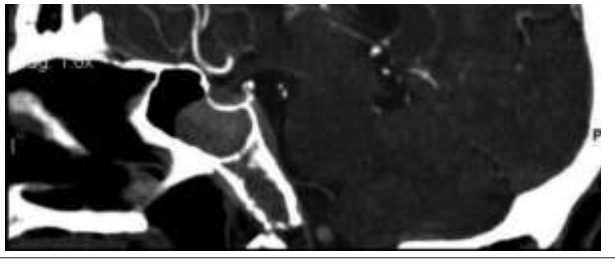

Figure 1.

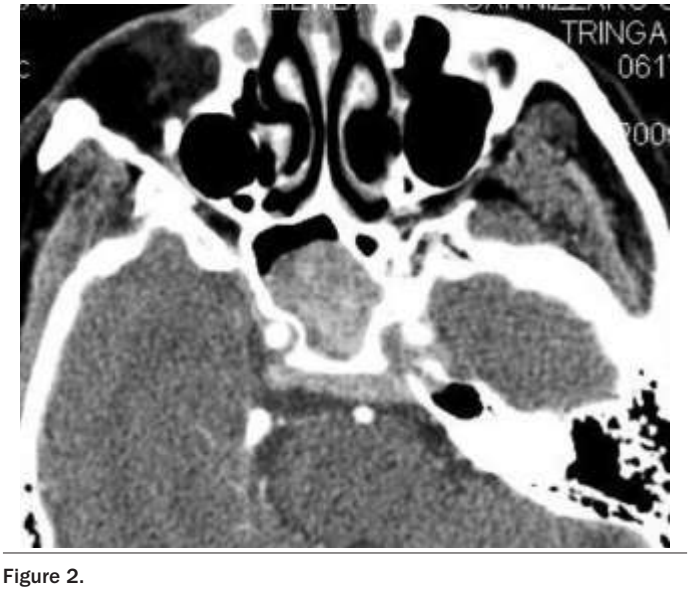

Figure 2.

In particular, he got a 2 weeks diplopia because of the left VI cn involvement, and he got a right side ocular paresis. A biopsy of the mass confirmed a plasmacytoma: a biopsy showed a diffuse infiltrate of plasma cells, with occasional atypical ones having enlarged nuclei, and multinucleated forms. Immunohistochemistry showed k light chain restriction and an immunoglobulin G immunophenotype. The plasma cells expressed CD79a, but not CD56. A comprensive work-up, including bone marrow biopsy, total-body skeletal survey, determination of urine Bence-Jones protein and serum myeloma protein was carried out in order to rule out multiple myeloma. He was referred for radiation therapy, receiving irradiation of 50 Gy for 6 weeks and had no recurrence of plasmacytoma or progression to multiple myeloma during follow up of 2 years.

PUB-10

DETECTION OF BCR-ABL ATP-BINDING DOMAIN MUTATIONS IN IMATINIB RESISTANCE CML PATIENT WHO RELAPSED AFTER BONE MARROW TRANSPLANT

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Bone marrow transplantation (BMT) is the most successful therapy for CML patients. Here we describe a CML patient who relapsed after bone marrow transplant. Patient was bone marrow transplanted but relapsed three years after successful BMT, showing signs of GvHD. Patient was treated with imatinib. After showing initial haematological and cytogenetic response, clinical resistance was noted in the patient. An ASO-PCR was epmloyed to detect BCR-ABL ATP-binding domain mutations. Mutations T1052 and C944T were detected (multiple mutations). This study shows that patients who relapse after bone marrow transplant and show GvDH can develop severe resistance to post-BMT Imatinib therapy.

PUB-11

STUDIES ON POST BONE MARROW TRANSPLANT (POST-BMT) IMATINIB RESISTANCE IN A CHRONIC MYELOID LEUKAEMIA (CML) PATIENT

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Bone marrow transplantation (BMT) has been proved to be the most successful therapy for CML patients. However, relapses have been reported in such patients and Imatinib therapy has been suggested for relapsing patients. Here we describe Imatinib resistance in a CML patient who underwent BMT in chronic phase, showed no signs of CML till three years after BMT but relapsed eventually, showing signs of GvHD. Following Imatinib therapy, patient showed haematological response for three months. Resistance to imatinib was noted after 6 months of treatment. Using ASO-PCR, multiple BCR-ABL ATP-binding domain mutations (T1052 and C944T) were detected in the patient who had advanced to accelerated phase of CML. Our findinds show that severe Imatinib resistance manifested by multiple mutations can be encountered in CML patients who relapse after bone marrow transplant.

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