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ABSTRACT BOOK

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ABSTRACT BOOK

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MAIN PROGRAM

MECHANISMS OF IMMUNOREGULATION OF ACUTE LEUKEMIA

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The initiation and development of acute leukemia is sustained by an immunosuppressive microenvironment where both innate and adaptive immune responses to cancer cells are profoundly deregulated. Several immunosuppressive mechanisms are operative resulting in the escape of tumor cells from natural immune control. Thus, a better understanding of the mechanisms governing the interplay between the microenvironment and cancer cells is mandatory for the development of innovative therapies. Indeed, modulation of the immune system by allogeneic stem cell transplantation and cellular adoptive immunotherapy has dramatically improved the long-term outcome of many patients whereas the targeting of specific immunosuppressive pathways has already entered the clinical arena with promising clinical results. A better knowledge of the immunologic phenotype of tumors and the role of check-points regulators, such as PD-1 and CTLA-4, IDO enzyme function and MSCs in the induction of immunological tolerance against tumors, is important to understand critical pathways for the manipulation of anti-tumor immune response. In particular, immune cell-rich tumors may optimally respond to targeting of negative immune regulators (e.g. lymphomas) while non T-cell infiltrated cancers may require the induction of inflammation to optimize immune responses. This translational research is instrumental, at the clinical level, for the development of novel molecules and strategies to counteract these tolerogenic pathways .In this presentation, some of the most important immunosuppressive mechanisms will be briefly reviewed as well as their potential role as targets of novel antitumor strategies.

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DETECTION OF CHRONIC MYELOID LEUKEMIA STEM CELLS BY FLOW-CYTOMETRY

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In chronic myeloid leukemia (CML) data suggest that relapse after tyrosine kinase inhibitors (TKIs) discontinuation is due to the persistence of leukemic stem cells (LSCs) intrinsically resistant to TKIs. Survival of CML LSCs may be the consequence of activation of several pathways BCR-ABL1 independent. qRT-PCR, the most sensitive assay to monitor disease status in CML patients, may be inappropriate to quantify residual quiescent CML LSCs that are transcriptionally silent. In CML, LSC supposedly reside within the CD34+/CD38-/Lin- fraction of the leukemic clone. However, normal hematopoietic SCs also exhibit this phenotype so that additional markers are required to discriminate CML LSC from normal SC. Until recently, exclusively the presence of the fused BCR-ABL gene by FISH in the purified LSC fraction, allowed researchers to identify Ph+ LSC.[1-4] Thus, up to date it has been quite difficult to identify and possibly quantify bone marrow residual CML LSCs during TKI treatment due to their small number and to a low-sensitive time-consuming technique based on cell separation. The possibility to easily detect and monitor the behavior of CML LSC during TKI treatment, in term of rate and timing of reduction and to correlate it with the molecular response would open a great opportunity to explore further the role of TKI in this disease.

Several surface markers such as CD123, CD25, CD117, IL1RAP, ST2, CD26 have been recently proposed to be upregulated on candidate CML stem cells, but few have so far been proven to separate Ph1 positive from negative LSCs. Recent studies have shown that within the $CD34^+/CD38^-$ population, the expression of IL1RAP may identify CML LSCs although the presence of this marker has been showing also in acute myeloid leukemia and normal hematopoietic SCs.[5] Landberg et al. have studied the expression of IL1RAP and CD25 on bone marrow isolated $CD34^+/CD38^-$CML cells. These authors proposed that IL1RAP expression, as a measure of LSC burden at diagnosis of CML predicts therapy outcome.[6] Other studies showed that CD25 antigen is expressed in human CML Leukemia Initiating Cells (LICs) and its expression positively correlates with CML disease progression.[7] Herrmann et al. described that $CD34^+/CD38^-/Lin^-$ CML LSC specifically co-express dipeptidylpeptidase IV (DPPIV= CD26) and that this enzyme disrupts LSC-niche interactions by degrading SDF-1.[8] Based on these data, CD26 appears to be a potential biomarker for the quantification and isolation of CML LSC, both in bone marrow and in peripheral blood.[9] As such Culen et al. recently quantified CD26+ LSCs bone marrow compartment in 31 CML patients at diagnosis and their number appears to correlate with response to TKIs treatment.[10] However, the feasibility to detect peripheral blood (PB) CML LSCs by flow-cytometry has never explored thoroughly.

Recently, we focused our research on testing and optimizing a new system to detect CML LSCs in PB by using a flow-cytometry multi-parameters approach. Firstly, the specificity of the $CD34^+/CD38^-/CD26^+$ antibody panel to identify CML LSCs has been validated by FISH analysis of PB sorted cells in CML patients. On the contrary, the flow-cytometry assessment of PB $CD26^+$ LSCs always scored negative when performed in normal subjects, non-CML patients undergoing G-CSF treatment and subsequent $CD34^+$

cell mobilization, patients with Ph- myeloproliferative disorders, including patients affected by myelofibrosis. We then checked for circulating CML LSCs in patients referring to several Italian Hematology centers, in first line treatment with any approved TKI. A total of more than 200 CML patients have been studied so far and in >70% of them we could detect residual CML LSCs. Clinical, molecular and flow-cytometry preliminary data are currently under elaboration. This study represents the first attempt to measure in a large cohort of CML patients residual circulating LSCs during TKIs treatment. In our hands PB LSCs flow-cytometry assay appeared feasible, specific and sensitive and thus suitable for routine monitoring. We just started a prospective study evaluating the behavior of PB CML LSCs during different TKIs treatment, and the monitoring of PB $CD26^+$ in CML pts that discontinued TKIs treatment is ongoing. The aim is to rule out the impact, if any, of a "stem cell response" in addition to the standard molecular response in the management of CML patients possibly to identify those pts candidates for a safe TKI discontinuation.

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HEREDITARY PREDISPOSITIONS TO MYELODYSPLASTIC SYNDROMES

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Myelodysplastic syndromes (MDS) are heterogeneous clonal hematopoietic disorders characterized by ineffective hematopoiesis, bone marrow dysplasia, and peripheral cytopenias. Familial forms of MDS have traditionally been considered rare, especially in adults; however, the increasing availability of somatic and germline genetic analyses has identified multiple multiple genes conferring inherited risks for MDS and/or acute myeloid leukemia (AML), including RUNX1, ANKRD26, DDX41, ETV6, GATA2, and SRP72 (Table 1). As these syndromes are increasingly appreciated in even apparently de novo presentations of MDS, it is important for hematologists to become familiar with these newly-described syndromes.

Familial platelet disorder with propensity to myeloid malignancy (FDP/AML). FDP/AML is an autosomal dominant familial MDS/AML syndrome caused by inherited mutations in the hematopoietic transcription factor RUNX1. Clinically, patients often present with life-long thrombocytopenia and aspirin-like functional platelet defects. The degree of thrombocytopenia is typically mild to moderate and can vary widely even within affected families from individuals with a normal platelet count, to severe thrombocytopenia, to childhood MDS/AML at the time of first evaluation. The lifetime risk of MDS or acute leukemia is estimated to be 35%–40%, with an average age at diagnosis of 33 years. Germline mutations in ETV6 and ANKRD26 genes are associated with autosomal dominant disorders similar to FPD/AML

Familial AML with Mutated DDX41. One of the most recently described inherited susceptibility loci for myeloid neoplasms, DDX41 germline mutations, located on chromosome 5q, are associated with autosomal dominant familial MDS/AML. DDX41 mutations result in an increased lifetime risk of myeloid neoplasms including MDS, AML, and chronic myeloid leukemia (CML), although notably after a long latency, with an average age of disease onset of 61 years. This average age at diagnosis falls within the expected age range of MDS/AML in the general population, thus it may be clinically difficult to distinguish patients with de novo MDS/AML from those with a germline predisposition due to a germline mutation in DDX41, and this syndrome may be particularly under-diagnosed.

Table 1. Familial myelodysplastic syndromes (MDS)/acute leukemia (AML) predisposition syndromes.

Syndrome	Gene	Inheritance	Hematological Malignancy	Other Associated Abnormalities
Platelet disorders with propensity to myeloid malignancies	*RUNX1, ETV6, ANKRD26*	AD	MDS/AML/	Thrombocytopenia, bleeding propensity, aspirin-like platelet dysfunction
Familial AML with mutated DDX41	*DDX41*	AD	MDS/AML, CMML	None
Familial MDS/AML with mutated GATA2	*GATA2*	AD	MDS/AML/CMML	Neutropenia, monocytopenia, MonoMAC syndrome, Emberger syndrome
Familial aplastic anemia with SRP72 mutation	*SRP72*	AD	MDS	Aplastic anemia
Familial AML with mutated CEBPA	*CEBPA*	AD	AML	None
Fanconi anemia	Complementation Groups	AR, X-linked	MDS, AML	Pancytopenia, macrocytic anemia, congenital malformations
Telomeropathies (dyskeratosis congenita)	*TERC, TERT,* others	AD, AR	MDS/AML	Macrocytosis, aplastic anemia, oral leukoplakia, dysplastic nails, lacy skin rash

AD, Autosomal dominant; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CMML, chronic myelomonocytic leukemia; AR, autosomal recessive.

Familial MDS/AML with Mutated GATA2 (GATA2 Deficiency). GATA2 deficiency is a clinically heterogenous predisposition to MDS. There are two distinct syndromic presentations that can be seen with this particular syndrome. Emberger syndrome describes GATA2 deficiency clinically characterized by primary lymphedema, sensorineural hearing loss, cutaneous/extragenital warts, and a low CD4/CD8 T-cell ration with a predisposition to MDS/AML. The MonoMac syndrome, is characterized by dendritic cells, monocytes, and B/NK cell deficiencies, leading to the development of atypical mycobacterial or fungal infections, pulmonary alveolar proteinosis, and MDS/AML predisposition. Individuals with GATA2 germline mutations are at significantly increased lifetime risk of MDS/AML; approximately 70% by a median age of onset of 29 years.

Familial Aplastic Anemia/MDS with SRP72 Mutation. Germline mutations in the ribonucleoprotein complex gene SRP72 (Signal Recognition Particle 72 kDa) have been identified as a rare cause of familial MDS and bone marrow failure. Two pedigrees with autosomal dominant MDS and aplastic anemia have been reported. In both families, MDS developed in adulthood. Given the rarity of these germline mutations, little is known regarding the incidence, lifetime risk for aplastic anemia (AA)/MDS and/or targeted clinical management guidelines of these families.

Recognizing patients with potential hereditary syndromes and

referring them for genetic evaluation and genetic counseling not only can provide valuable insights for treatment of their disease but also education, risk assessment, and psychosocial support for these individuals and their family members.

NOVEL ERYTHROPOIESIS STIMULATING AGENTS. EXPERIMENTAL EVOLUTION, MECHANISM OF ACTION AND INTRODUCTION IN CLINICAL PRACTICE IN ONCOLOGICAL AND NON-ONCOLOGICAL HEMATOLOGY

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Here we describe novel erythropoiesis stimulating agents. Different strategies are used to optimize erythropoiesis: (i) reduction in globin chain imbalance via HbF induction; (ii) Jak2 inhibition; (iii) modulation of GDF11 via activin receptor traps; (iv) iron restriction via hepcidin's signal modulation; (v) manipulation of molecular chaperones and (vi) potentiation of endogenous antioxidant system.
GDF11 is a member of the bone morphogenetic protein (BMP) family and of the TGF-beta superfamily. It is also a ligand of the activin receptor-II trap ligands A and IIB (ActRIIA and ActRIIB). These molecules form complexes with additional receptors that regulate gene expression primarily by activating the SMAD2/3 subfamily of intracellular effectors.
Ligand traps are molecules that inhibit signaling by binding ligands and sequestering them away from their receptors. ActRIIA and ActRIIB are recognized by several ligands, including GDF11, and have been involved in a variety of physiological functions, including bone homeostasis and age-related bone loss. The trap ligand ACE-011 (sotatercept) was made by fusing the extracellular domain of ActRIIA to the Fc domain of human immunoglobulin G1 (IgG1) and was originally developed to improve bone mineral density (BMD) in menopausal women.[1] In a phase I clinical trial in postmenopausal women with osteoporosis, ACE-011 increased BMD and interestingly (and unexpectedly) increased Hb values .[1] Further investigations into ACE-011 and another trap ligand targeting ActRIIB (ACE-536, luspatercept), in mouse models of myelodysplastic syndromes (MDS) and beta-thalassemia showed significant improvement of the anemia.[2-4] It has been suggested that the mechanism of action of these drugs in both of these disorders is mediated by targeting GDF11. This decreases Smad2/3 activation in erythroid progenitors, and ultimately improves erythroid maturation and RBC production.[2-4] Studies have demonstrated that GDF11 is overexpressed in the spleen and erythroid cells of thalassaemic animals (and in the serum of patients with thalassaemia) and its inhibition in mouse models of anemia with ineffective erythropoiesis restores normal erythropoietic differentiation and alleviates anemia. Overexpression of GDF11 may be responsible for the ineffective erythropoiesis associated with beta-thalassaemia. GDF11 itself also inhibits erythroid maturation in mice *in vivo* and *ex vivo*, while expression of GDF11 and ActRIIB in erythroid precursors decreased progressively with maturation, suggesting an inhibitory role for GDF11 in late-stage erythroid differentiation.
Clinical trials are ongoing:
- a phase-1-2 of ACE-011 (sotatercept) in patients with Transfusion-Dependent Diamond Blackfan Anemia (NCT01464164);
- a phase-3 of ACE-536 (luspatercept) in patients with Beta thalassemia syndromes (NCT02604433);
- a phase-3 of ACE-536 (luspatercept) in patients with Very Low, Low, or Intermediate Risk Myelodysplastic Syndromes (NCT02631070)

Alteration of protein homeostasis networks by pharmacologic manipulation of chaperone machinery and protein degradation pathways represents a novel target for decreasing ineffective erythropoiesis. Normal human erythroid maturation requires a transient activation of caspase-3 in the later stages of maturation. Chaperone heat shock protein 70 (HSP70) is constitutively expressed in erythroblasts and, at later stages of maturation, translocates into the nucleus and co-localizes with GATA-1, protecting it from caspase-3 cleavage. This ubiquitous chaperone participates in the refolding of proteins denatured by cytoplasmic stress, thus preventing their aggregation and thus playing an anti-apoptotic role. During the maturation of human TDT erythroblasts, HSP70 interacts directly with free alpha-globin chains. As a consequence, HSP70 is sequestrated in the cytoplasm and GATA-1 is no longer protected. This results in arrest of end-stage maturation and apoptosis. Transduction of a nuclear-targeted HSP70 mutant or a caspase-3-uncleavable GATA-1 mutant restores terminal maturation of beta-thalassemic erythroblasts, which may provide a rationale for the development of targeted therapies for beta-thalassemia.[5]
Potentiation of endogenous antioxidant system via AG348, a novel, first-in-class PiruvatoKinasi-Receptor, showed to reduce ineffective erythropoiesis increasing Hb level in beta thalassemic mice (European Hematology Association, 2016).

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NK ALLOREACTIVITY AS A PLATFORM FOR TARGETING MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) treatment in adult patients is based on intensive chemotherapy. Complete remission (CR) rate after chemotherapy ranges from 60 to 85%, but the overall survival (OS) is 40% in patients younger than 60 years, and falls to 10% in those older than 60 years.[1] The main reason for such dismal clinical outcome is that many patients, who achieve CR after chemotherapy, still harbor a minimal residual disease (MRD), which eventually leads to relapse and progression, being resistant to further pharmacological treatments. MRD treatment is allogeneic stem cell transplantation (SCT), but such approach has several limitations, especially for over 60-years old patients. Therefore, new strategies addressing relapse prevention, especially in patients not eligible for allogeneic SCT, are highly warranted.

Human NK cells are a subset of PB lymphocytes defined by the expression of CD56 or CD16 and the absence of the T-cell receptor (CD3).[2] They recognize and kill transformed cell lines in an MHC-unrestricted fashion and play a critical role in the innate immune response. Several studies demonstrated that NK function, which is distinct from the MHC-restricted cytolytic activity of T cells, may be relevant for the immune control of tumor development and growth.[1] Although NK cell killing is MHC-unrestricted, NK cells display a number of activating and inhibitory receptors that ligate MHC molecules to modulate the immune response.[3] NK cell recep-

tors that recognize antigens at the HLA-A, -B, or -C loci are members of the immunoglobulin super family and are termed killer immunoglobulin receptors or KIRs. Engagement of these NK cell receptors results in stimulation or inhibition of NK cell effector function, which ultimately depends on the net effect of activating and inhibitory receptors.

Data from haploidentical T-cell depleted transplantation suggest that KIR mismatch with tumor MHC may significantly impact on tumor cell killing, particularly in AML.[4] In fact, these studies show that AML patients with KIR ligand mismatch are significantly protected against leukemia relapse. In particular, high risk AML patients with a KIR-ligand mismatch in the graft-versus-host (GVDH) direction had a relapse rate of 0% compared to KIR-ligand matched patients who had a relapse rate of 75%. Given these results, haploidentical KIR-mismatch NK cells administered to AML patients as cell-based infusion may induce NK cell-mediated killing of leukemia cells resulting in the elimination of residual disease in high risk AML patients.

In a pioneering study, Miller et al demonstrated that up to 1.5 $x10^7$/haploidentical NK cells/Kg can be safely infused in AML and cancer patients following Fludarabine/Cyclophosphamide (Flu/Cy) immunosuppressive chemotherapy and, in some cases, clinical responses without GVHD had been observed.[5] In particular, in this study 19 poor risk AML patients were reported who had received a cell population containing a median of $8.5\pm0.5x10^6$ and $1.75\pm0.3x10^5$ NK and T cells, respectively. Five out of 19 patients achieved CR. NK cells infusion was well tolerated and hematological and non-hematological toxicity were mainly related to the immunosuppressive regimen and IL-2 administration. Our group published the clinical results of haploidentical NK cell infusion in adult AML patients with relapsing/refractory disease (6). No specific NK cell-related toxicity was observed. Donor-versus-recipient alloreactive NK cells were demonstrated in vivo by the detection of donor-derived NK clones and adoptively transferred NK cells were alloreactive against recipient's leukemic cells. Subsequently, we extended our approach to a cohort of elderly AML patients, who achieved CR after induction/consolidation chemotherapy, as part of consolidation program.[7] Seventeen AML patients, in first morphological CR (3 patients showed molecular disease) (median age 64 years, range 53-73) received highly purified CD56+CD3- NK cells from haploidentical KIR-L mismatched donors after fludarabine/cyclophosphamide immunosuppressive chemotherapy, followed by interleukin-(IL) 2. With a median follow-up of 22.5 months (range, 6-68 months), 9/16 evaluable patients (56%) are alive disease-free, whereas 7/16 (44%) relapsed with a median time to relapse of 9 months (range, 3-51 months). Among relapsed patients, 2 individuals showed a very prolonged CR phase of 24 and 51 months, respectively, in absence of any concomitant anti-leukemia treatment. Three patients, treated with molecular disease, achieved molecular CR lasting 9 and 4 months in 2 cases and 8+ months in the third patient. Adoptively transferred NK cells were alloreactive against recipient's cells, including leukemia. More importantly, higher frequencies of NK alloreactive clones in the donors statistically correlated with better clinical outcome

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INFLAMMATION AND CANCER

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Macrophages are key orchestrators of chronic inflammation. They respond to microenvironmental signals with polarized genetic and functional programmes. M1 macrophages which are classically activated by microbial products and interferon-γ, are potent effector cells which kill microorganisms and tumours . In contrast, M2 cells, tune inflammation and adaptive immunity; promote cell proliferation by producing growth factors and products of the arginase pathway (ornithine and polyamines); scavenge debris by expressing scavenger receptors; promote angiogenesis, tissue remodeling and repair. M1 and M2 cells represent simplified extremes of a continuum of functional states. Available information suggests that someTAM are a prototypic M2 population. Polarization of phagocytes sets these cells in a tissue remodeling and repair mode and orchestrate the smouldering and polarized chronic inflammation associated to established neoplasia. Intrinsic metabolic features and orchestration of metabolism are key components of macrophage polarization and function. Recent studies have begun to address the central issue of the relationship between genetic events causing cancer and activation of protumour, smouldering, non-resolving tumour-promoting inflammation. New vistas have emerged on molecules associated with M2 or M2-like polarization and its orchestration in cancer. Recently, proof-of-principle has been obtained that targeting TAM can be beneficial in human cancer. Moreover, complement has emerged as a key component of tumor-promoting inflammation.

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MACROPHAGES AND BONE MARROW MICROENVIRONMENT SUPPORT PROGGESSION OF LYMPHOPROLIFERATIVE DISEASES

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Lymphoproliferative diseases of B cell lineage display a bone mar-

row (BM) microenvironment that plays a critical role in favoring tumor cell survival, proliferation, escape from immunosurveillance and development of drug-resistance (DR). The microenvironment presents a high variability as the composition of cells, including immune and inflammatory cells, blood and lymphatic vascular networks, cytokines, growth factors and extracellular matrix.[1] In lymphoproliferative diseases, the BM microenvironment is very complex since it is formed by a non-cellular compartment, *i.e.*, the extracellular matrix proteins, and soluble factors (cytokines, growth factors, chemokines), and by a cellular one including hematopoietic (myeloid cells, macrophages, T lymphocytes, B lymphocytes, NK cells) and non-hematopoietic cells (fibroblasts, osteoblasts, osteoclasts, endothelial cells, pericytes, mesenchymal stem cells). All these cells form a specialized niche that plays a key role for multiple myeloma (MM) onset, progression and DR.[2]

A relevant number of studies unveil the role of tumor-associated macrophages (TAMs) as important key players in this microenvironment. TAMs are a heterogeneous population originating from blood monocytes, which are able of immunostimulation and immunosuppressive activities. They are grouped into "classic activated" TAMs or M1; and "alternatively activated" TAMs or M2. The first one act as soldiers defending the host from viral and microbial infections, and hence activate the immune response; the second one control the inflammatory response by down-regulating M1 cell-mediated functions, promote angiogenesis, and suppress adaptive immunity.[3] Immunohistochemistry and electron microscopy analysis reveal that macrophages increase significantly in B-cell non-Hodgkin's lymphomas and stimulate the induction of angiogenesis through releasing their angiogenic factors and promoting tumor progression.[4]Also in classic Hodgkin's lymphoma it has been well demonstrated that an increased number of tumor-infiltrating macrophages is associated with shortened survival.[5] TAMs are deeply implicated in the biology, cell proliferation and progression in chronic lymphocytic leukemia (CLL) cells[6] and in MM.

The MM-associated macrophages are recruited and activated by plasma cells (PCs) secreting vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), and induce inflammatory cells to release their own cytokines and growth factors, i.e. VEGF, FGF-2, tumor necrosis factor- (TNF-), interleukin-6 (IL-6), IL-12, IL-10, and stromal cell-derived factor-1 (SDF-1).[7] FACS analysis of BM biopsies shows higher number of $CD68^+$ macrophages in patients with active MM compared to those with MGUS.[8] We demonstrated that BM macrophages stimulated with VEGF and basic FGF (FGF-2) mimic endothelial cells (ECs) behavior keeping their CD14 and CD68 lineage markers and contribute to build neovessels in active MM through vasculogenic mimicry.[8] Exposure of BM macrophages to bortezomib and zoledronic acid impacts their angiogenic and vasculogenic properties,[3] suggesting that BM macrophages may be considered an important target of both drugs in MM patients.

BM-macrophages are also able to activate fibroblasts.[9] Cancer-associated fibroblasts (CAFs) are a relevant cell population in the BM microenvironment that increase and parallel with MM clinical stages and promote an inflammatory condition associated with cancer growth and angiogenesis.[10] Indeed, in patients with active MM, CAFs produce increased levels of transforming growth factor-beta (TGF-), IL-6, SDF-1 , insulin-like growth factor-1 (IGF-1), VEGF and FGF-2 in contrast with MGUS patients suggesting a supportive role for MM progression.

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MULTIPLE MYELOMA-INDUCED OSTEOLYSIS: ROLE OF INFLAMMATION

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The critical role of inflammation in cancer development has been described since 1863.[1] The inflammatory environment enhances cell proliferation and survival and increases the angiogenesis, thereby promoting tumor development.[2] Moreover, age-related disruption of the pro and anti inflammatory cytokine homeostasis induces a chronic state of low-grade inflammation that contributes to age-related pathologies, as Multiple Myeloma (MM).[3] MM is a plasma cell (PC) malignancy characterized by a tight relationship between tumor cells and those of the microenvironment including endothelial cells, osteoclasts (OC) and osteoblasts (OB).

Among the clinical features of MM patients, osteolysis is the hallmark of the active disease as compared to patients with indolent monoclonal gammopathies as the monoclonal gammopathy of uncertain significance (MGUS) and the smoldering MM (SMM). Osteolysis is due to an unbalanced and uncoupled bone remodelling process. The increased OC formation and activity in MM is mainly induced by the upregulation of the receptor activator of nuclear factor-kappa B ligand (RANKL) and a down regulation of its decoy receptor osteoprotegerin.[4] Interestingly, several studies showed that also T lymphocytes are involved in MM osteoclastogenesis. They expressed high levels of RANKL and released pro-osteoclastogenic factors, such as IL-3.[5] More recently, it has been demonstrated an increase of Th17 phenotype in the BM of osteolytic MM patients able to produce IL-17, a cytokine involved in RANKL upregulation by the microenvironment. Interestingly our group showed that the chemokine (C-C motif) ligand 20 (CCL20), the main chemokine involved in Th17 recruitment, is overexpressed in the MM bone microenvironment.[6] Consistently we recently reported that BM CCL20 levels correlated with the presence of osteolytic lesions in MM patients.[7] Interestingly, CCL20 was also overexpressed in patients with Langherans cell histiocytosis that share similar osteolytic lesions to myeloma patients with the involvement of dendritic cells.[8] On the other hand, dendritic cells (DC) are significantly altered in MM patients(9). The dysregulation of both OC and DC suggests the pivotal role of monocytes, their common precursors, among MM bone microenvironment cells. Recently it has been reported that monocytes themselves may produce cytokines such as Activin A involved in both OC formation and OB inhibition.[10] Because the pivotal role of monocytes, the aim of our recent study was to analyze the immunophenotypic and transcriptional profiles of BM $CD14^+$ cells across the different types of monoclonal gammopathies, in order to identify alterations potentially involved in the pathophysiology of MM-induced osteoclastogenesis.

Monocytes can be classified in different subsets, based on the

expression of two surface molecules, CD14 and CD16. $CD16^+$ monocytes show a pro-inflammatory phenotype, with an enhanced production of TNF-alpha and IL-12 and a more efficient activity as antigen presenting cells. We showed that MM patients have a higher number of $CD14^+CD16^+$ cells as compared to MGUS. Moreover, after sorting the two populations according to CD16 expression, we found that $CD14^+CD16^+$ cells in MM patients are more pro-osteoclastogenic in ex vivo cultures, as compared to the $CD14^+CD16^-$ cells. Comparing symptomatic vs asymptomatic patients with monoclonal gammopathy, we identified 61 genes (37 up-regulated and 24 down-regulated). Among the overexpressed genes, there are factors involved in the immune response, cell migration and chemotaxis, and in the osteoclastogenic differentiation, such as CXCL10 and IL21R. IL21R was expressed at high intensity in the pro-osteoclastogenic monocyte subpopulation $CD14^+CD16^+$. In line with these data, we found a significant higher activation of STAT3 signaling (the activated pathway down-stream IL- 21R) in $CD14^+$ cells from MM patients, as compared to SMM and MGUS. Thereafter we overexpressed IL21R in $CD14^+$ cells from healthy donors with a lentiviral vector and we observed an increased osteoclastogenesis from these cells, compared to cells infected with an empty vector. Consistently, the presence of a STAT3 inhibitor, known to block IL-21R signaling, significantly reduced the OC formation in IL21R overexpressing $CD14^+$ cells. In addition, we showed that blocking IL-21R signaling in $CD14^+$ cells from MM patients, significantly inhibited OC formation independently by the presence of IL-21, further supporting the role of IL21R overexpression in the MM-induced osteoclastogenesis.

Overall, our data and those of the literature support the notion that a pro-inflammatory profile of BM $CD14^+$ cells is involved in osteolysis in MM. Among the genes overexpressed in BM monocytes of MM patients, we highlighted for the first time IL21R and demonstrated its role in the increased osteoclastogenesis, suggesting that IL-21R signaling could be a potential new therapeutic target for MM bone disease.

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IMMUNOGENIC CELL DEATH AND TOLERANCE INDUCTION: FOCUS ON INDOLEAMINE 2,3-DIOXYGENASE

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Acute myeloid leukemia (AML) is a clonal disorder, sprouting from a population of leukemic stem cells. In the last years, major strides have been made in the understanding of AML biology, but these preclinical advances have weakly impacted on the clinical outcome of AML patients, whose prognosis is overall largely unsatisfactory.[1]

The cancer cell death induced by some chemotherapeutic agents, especially anthracyclines, such as daunorubicin (DNR), is highly immunogenic and results in the efficient cross-priming of anti-tumor T cells through dendritic cells (DCs).[2] Such process, named immunogenic cell death (ICD), is characterized by intracellular and pericellular modifications, including the translocation of calreticulin (CRT) from nucleus to cell surface as well as the extracellular release of high mobility group box 1 (HMGB1) and adenosine triphosphate (ATP), which favor the presentation of tumor antigens to T cells by DCs.[3] Although a large body of evidence supports the role of ICD both in solid tumors and in leukemias,[4] including AML, some reports also indicate that anticancer drugs, while triggering ICD, induce expansion of regulatory T cells (Tregs).[6]

DCs are key regulators of adaptive immunity, promoting or suppressing T-cell responses.[6] One of the suppressive mechanisms involves the expression of indoleamine 2,3-dioxygense (IDO),[7] which degrades the essential amino acid tryptophan into kynurenine. Along with its enzymatic function (Figure 1), IDO plays a major role in the induction of T-cell tolerance through the expansion of Tregs. In particular, IDO-mediated induction of Tregs is a major mechanism by which AML cells hamper the efficient activation of anti-leukemia immune response by creating an immunosuppressive microenvironment.[8,9]

In the present study, we report that ATP released from dying AML cells during ICD has ambivalent immunological effect resulting in both activating anti-leukemia immune response and inducing Tregs accumulation through IDO1 up-regulation in DCs. Indeed, *ex vivo* analysis of AML patients, undergoing DNR-based chemotherapy, revealed a concomitant increase of leukemia-specific IFN-γ producing $CD4^+$ and $CD8^+$ T cells, and Tregs. Interestingly, $CD8^+$ IFN-γ-producing T cells showed an exhausted phenotype and defective cytotoxic function. These data were paralleled with the results obtained from *in vivo* mouse model, where DNR treatment increased plasma levels of activatory (IFN-gamma, IL-1beta, TNF-alpha, IL-12) and tolerogenic (IL-10) cyotkines. Interestingly, tumour-infiltrating $CD8^+$ T cells after DNR treatment showed an exhausted phenotype, were defective in TNF-alpha production and an increase in IFN-γ-producing Tregs was observed. In the attempt to give an explanation of these results, DNR treatment increased ATP release from AML cells *in vitro* and *in vivo*. However, in DNR-treated mice we observed a significant increase of $CD11c^+$ mature DCs which express IDO1 in tumor infiltrate. *In vitro*, loading of DNR-treated AML cells into DCs resulted in increased maturation, but also in IDO1 induction, which is involved in Tregs expansion. Interestingly, we showed that extracellular ATP is directly involved in DCs maturation and IDO1 expression via purinergic receptor P2Y11.

In AML a better understanding of the interplay between ICD and immune tolerance may provide the rationale for developing novel immunological therapeutical strategies, which may optimize the immunogenic effect of chemotherapy by contrasting the concomitant induction of tolerogenic pathways. Strategic combination of immunotherapy, such as checkpoint and IDO inhibitors, and chemotherapy can impact on AML microenvironment, thus resulting in effective and durable immune response. Such approach may translate into clinical benefit for AML patients.

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IMMUNE SUPPRESSION MECHANISMS IN MULTIPLE MYELOMA: ROLE OF IMMUNE CHECKPOINTS

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A growing body of evidence indicates that tumor cells exploit immune checkpoints to withstand immune recognition and onslaught. Pre-clinical findings, corroborated by initial results of clinical studies, indicate that immune checkpoint blockade is a promising strategy to harness anti-tumor immune responses and improve the clinical outcome of patients with hematological malignancies. Multiple Myeloma (MM) is a prototypic disease in which immune checkpoints significantly contribute to the immune suppressive contexture that myeloma cells establish in the bone marrow (BM) in cooperation with regulatory T cells (Tregs), myeloid derived suppressor cells (MDSC), and BM stromal cells (BMSC). Among the immune cells victimized by these interactions are Vγ9V 2 T cells. These are non-conventional T cells halfway between innate and adaptive immunity with a natural inclination to react against malignant B cells, including myeloma cells.[1] Vγ9V 2 T cells are equipped with a peculiar array of receptors for stress-induced self-ligands and a unique TCR-dependent recognition ability of phosphoantigens (pAgs) generated in the mevalonate (Mev) pathway. BM Vγ9V 2 T cells are anergic to pAg stimulation[2-4] and we have already shown that the programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) immune checkpoint contributes to Vγ9V 2 T-cell dysfunction.[4] This is an early event that can be already detected in individuals with monoclonal gammopathy of undetermined significance (MGUS) and not fully reverted even when MM patients achieve clinical remission after autologous stem cell transplantation (auto-SCT).[4]

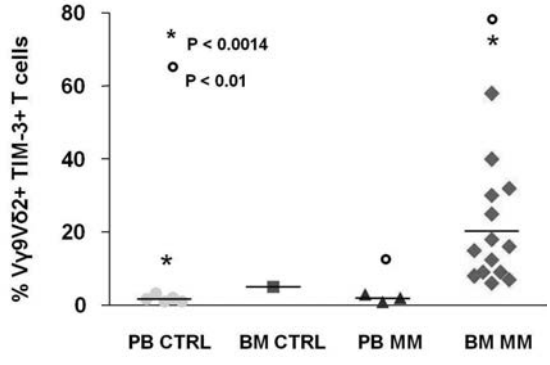

Figure 1. Increased TIM-3 expression in BM Vγ9Vδ2 T cells. Percentages of baseline TIM-3 expression on Vγ9Vδ2 T cells in the PB and BM of healthy donors (CTRL) and MM patients at diagnosis.

Anti-PD-1 treatment partially recovers the ability of BM Vγ9V 2 T cells to proliferate and exert cytotoxic activity after pAg stimulation.[4] Unfortunately, early studies based on single-agent PD-1 blockade have fallen short of clinical expectations in MM,[5,6] and several strategies are under consideration to implement the clinical efficacy such as the association with lenalidomide, and concurrent tumor vaccination.[7] PD-1/PD-L1 pair is not the only immune checkpoint involved in tumor escape. TIM-3/Gal9, BTLA/HVEM, and LAG3/MHC class II pairs are other immune checkpoints suppressing anti-tumor responses in solid tumors and haematological malignancies. Our results indicate that TIM-3 is significantly upregulated in BM Vγ9V 2 T cells from MM patients at diagnosis (Figure 1). Unlike healthy immune cells, the attempt to activate functionally exhausted immune cells can further compromise their anti-tumor functions. pAg stimulation of PD-1+ BM Vγ9V 2 T cells further increase PD-1 expression[4] and also increases TIM-3 expression. Interestingly, TIM-3 up-regulation is even more pronounced than PD-1 up-regulation in BM Vγ9V 2 T cells and it occurs also in peripheral blood (PB) Vγ9V 2 T cells from anergic (NR) MM patients. As expected, pAg-induced TIM-3 up-regulation (Figure 2, left panel) is closely related to the defective pAg-indced Vγ9V 2 T-cell proliferation (Figure 2, right panel). These data indicate that the attempts to activate Vγ9V 2 T cells in the absence of other appropriate immune interventions may further deteriorate their immune competence status.

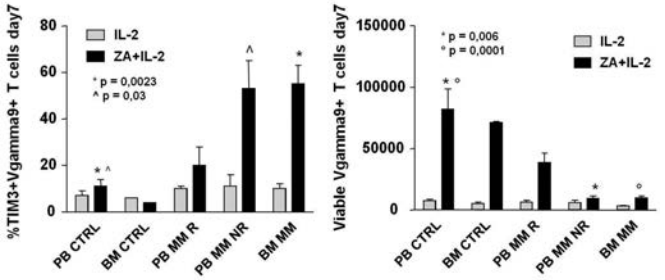

Figure 2. TIM-3 expression (left panel) and total counts of viable Vγ9Vδ2 T cells per well (right panel) after 7-day stimulation of PBMC and BMMC from CTRL and responder (R) and non-responder (NR) MM patients with IL-2 or ZA+IL2.

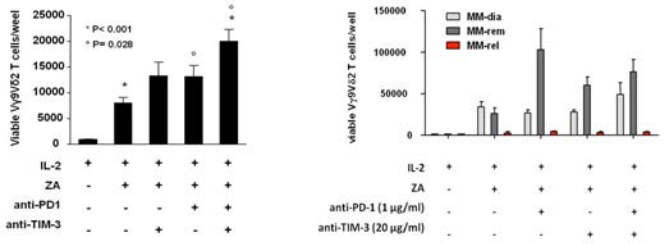

Figure 3. Left panel: proliferation of pAg-stimulated BM Vγ9Vδ2 T cells in the presence of anti-PD-1 and /or anti-TIM-3 blockade. Right panel: proliferation of pAg-stimulated PB Vγ9Vδ2 T cells from MM at diagnosis (n=5), MM in remission (n=6) and MM in relapse (n=5), in the presence of anti-PD-1 and /or anti-TIM-3 blockade.

As already observed for PD-1 blockade,[4] also TIM-3 neutralization is able to partially recover pAg-induced Vγ9V 2 T-cell proliferation. The best recovery is however obtained when pAg stimulation is carried out in the presence of concurrent PD-1 and TIM-3 blockade (Figure 3, left panel). As reported in Figure 3 (right panel), the effect of immune checkpoint blockade changes in different phases of the disease: the most significant recovery of Vγ9V 2 T-cell proliferation is observed after PD-1 blockade in MM remission (MM-rem); the anergy of Vγ9V 2 T cells from MM in relapse (MM-rel) is refractory to immune checkpoint blockade, whereas Vγ9V 2 T cells from MM at diagnosis (MM-dia) show intermediate responses and perform better when stimulated in the presence of concurrent anti-PD-1/anti-TIM-3 blockade.

In conclusion, our data suggest that immune checkpoint blockade combinations could be a potentially promising approach for cancer immunotherapy in MM patients.

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RELATED SIGNATURE, WITH FUNCTIONALLY RELEVANT ALTERATIONS IN HLA CLASS II ANTIGEN PRESENTATION AND T CELL COSTIMULATION

C. Toffalori[1], M. Riba[2], L. Zito[1], O. Oliveira[1,3], G. Bucci[2], M. Barcella[4], O. Spinelli[5], R. Greco[6], L. Crucitti[6], N. Cieri[3,6], M. Noviello[3], D. Cittaro[2], D. Lazarevic[2], M.G. Carrabba[6], M. Bernardi[6], J. Peccatori[6], C. Barlassina[4], E. Stupka [2], G. Tonon[2], A. Rambaldi[5], C. Bonini[3], K. Fleischhauer[1,7], F. Ciceri[6], L.Vago[1,6]

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Despite the considerable progress achieved during the last decades in the cure of acute myeloid leukemia (AML), mainly due to improved knowledge of its genetic asset and wider use of allogeneic hematopoietic stem cell transplantation (HSCT), post-transplantation relapses remain frequent and largely incurable. We demonstrated that a frequent mechanism of relapse after partially-incompatible HSCT is the genomic loss in leukemic cells of the HLA haplotype mismatched between patient and donor[1,2] providing a proof-of-principle for the hypothesis that post-transplantation relapses might represent the expression of mechanisms of leukemia immune evasion from the donor immune system. During the presentation original findings regarding new mechanisms of post-transplantation relapse will be presented. In particular, it will be shown how the deregulation of immune-related processes, and in particular of the pathways involved in T cell-mediated target recognition and costimulation, represents a distinctive feature of AML relapses after allogeneic HSCT. These observations point out the relevance of identifying and classifying patient-specific mechanisms of leukemia immune evasion and relapse after allogeneic HSCT, in order to personalize therapeutic strategies.

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DEVELOPMENT OF AN INNOVATIVE TARGETED THERAPIES WITH T LYMPHOCYTES AND BISPECIFIC ANTIBODIES

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Several approaches have been developed in recent years to redirect either endogenous T cells or *in vitro* expanded T cells to specifically kill tumour cell targets. In the first case, bispecific antibodies can be used, such as the BiTEs antibodies, exemplified by CD3xCD19 blinatumomab, which activates and induces the proliferation in vivo of endogenous cytotoxic T cells and redirects them to kill $CD19^+$ leukaemias and lymphomas. Although this drug has shown significant efficacy in clinical trials, some patients do not respond or relapse early following therapy. A major cause of resistance or relapse may be the low number and or anergic state of endogenous T cells in some patients, suggesting a need for improvements. In the other approach, peripheral blood T cells derived from a donor or from the patients themselves are expanded in vitro and genetically modified with chimeric antigen receptors (CAR) to redirect their cytotoxic activity efficaciously towards the tumour after infusion. In this case one major problem encountered is the cost and potential danger of genetically modifying T cells for in vivo use, due to the possible long term permanence of the modified cells or potential transformation in vivo. We are therefore developing novel methods to obviate to some of the above mentioned problems. In particular we have developed a novel GMP-compliant method to expand polyclonal activated T cells from patients with B-ALL or B-NHL, while at the same time eliminating the $CD19^+$ leukaemic cells in the samples, to be used in an autologous setting alone or in combination with bispecific antibodies, such as blinatumomab (Golay *et al.* 2014, J. Immunol. 193(9):4739-47). The methods use blinatumomab and rhIL-2 for expansion and the final activated T cell product are called BET (blinatumomab expanded T cells). Thus large number of disease free BET can be expanded in vitro from CLL or B-NHL patients for immunotherapeutic purposes. In parallel studies we have developed a novel tetravalent Fc-bearing bispecific antibody format, capable of redirecting activated T cells towards the tumour targets (Golay *et al.* J. Immunol. 2016; 196(7):3199-211). The new BsAb efficiently redirects T cells to kill tumour targets *in vitro* and *in vivo*.

Progress along these lines of research will be presented.

MODIFICATIONS OF VON WILLEBRAND FACTOR IN ESSENTIAL THROMBOCYTHEMIA

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Essential thrombocythemia (ET) is a myeloproliferative neoplasm characterized by increased platelets and prevalent thrombosis. An acquired Von Willebrand factor (VWF) disease in ET has been associated with extreme thrombocytosis or, more rarely with bleedings. Whether and how VWF is modified in ET patients remains unclear. In this investigation different VWF- and platelet- associated parameters in ET patients treated according to current recommendations were extensively studied. Sixty-nine ET patients under low dose aspirin (M=29; median age: 62[48-70]yrs, platelets: 432[337-620]x10^3/μL), 69 matched controls and 10 reactive thrombocytosis (RT) were enrolled in the study. VWF:antigen (Ag), activity (act), electrophoretic patterns, VWF:propeptide , plasma glycocalycin (GC), glycoprotein-V (GpV), ADAMTS-13, elastase, C- reactive protein and serum thromboxane (TX)B_2 were measured.. In ET, VWF:Ag was significantly increased by 31±13% vs. controls ($p<0.01$), independently from blood groups, while VWF:act was reduced by 21±12% vs. controls and by 50±24% vs. RT ($p<0.01$). The VWF:act/VWF:Ag ratios in ET were reduced by 35±17% versus controls and RT ($p<0.001$) and significantly associated with immature or total platelet counts, GC, GpV, and TXB_2. In multivariable analysis, only GC was associated and inversely predicted ET VWF:act/VW:Ag ratios (β =-0.42, p=0.01). By SDS-agarose electrophoresis analyses, high-molecular-weight VWF multimers were variably reduced with atypical cleavage bands in ET only. VWF:propeptide and ADAMTS-13 levels were normal among ET patients. Platelet-associated ADAM-10 and ADAM-17 hydrolyzed VWFm *in vitro* showed patterns similar to ET samples. In ET patients with controlled platelet counts, the VWF:act/Ag is decreased and predicted by GC, a product of platelet activation. ADAMs may be involved in the process of uncontrolled VWF proteolysis, causing a net decrease of high molecular weight multimers. *In vivo* platelet activation, which characterizes ET, might contribute to VWF alterations.

THROMBIN GENERATION IN CANCER

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Venous thromboembolism (VTE) is one of the most common complications observed in cancer patients and different clinical and biological risk factors contribute to the overall thrombotic risk in this disease.[1] The hypercoagulable state developing during cancer progression has a multifactorial origin and the tumor tissue capacity to interact with and activate the host hemostatic system plays an important role.[2]

Activation of blood coagulation cause an increase in thrombin generation and fibrin formation. Thrombin is a pleiotropic enzyme: contributes to fibrin formation and platelet aggregation during vascular hemostasis, but it can also trigger cellular events through protease-activated receptors, PAR-1 and PAR-4. Furthermore, many studies provided evidence that thrombin can regulate every step of cancer dissemination. Therefore, the measurement of thrombin generation (TG) in cancer might represent a practical tool to quantify the combined and cumulative effect of the different and multiple factors contributing to cancer-associated coagulopathy and to assess VTE risk[3].

Different methods are available for TG evaluation, and, among these, the Calibrated Automated Thrombography (the CAT assay) that employs a fluorogenic thrombin substrate is the most utilized [4,5]. The CAT assay measures the generation of thrombin over time in platelet free plasma supplemented with phospholipids or in platelet-rich plasma. Triggering is done by picomolar concentration of Tissue Factor (TF), even though other triggers can be used.[3] The result is a TG curve (the thrombogram) characterized by different parameters, including the lag-time (time until thrombin burst), the peak of thrombin generation, time to peak of thrombin, the total amount of thrombin generated (ETP, endogenous thrombin potential) and the velocity of thrombin generation. The ETP is the most utilized parameter, and its elevation characterizes patients at higher thrombotic risk, while lower ETP levels are present in patients with congenital and acquired coagulation factor deficiencies. Despite the CAT assay gives reproducible results when performed in a single laboratory, standardizations of the TG assay is required to minimize inter-laboratory variations.[6] The use of identical equipment, standardized reagents, selected reference plasma for normalization of results and the same test procedure, has significantly reduced the assay variability among different laboratories.[7]

Numerous studies found elevated TG potential in patients with different solid or hematological cancer. [8,9] Thrombosis has a significant impact on the morbidity and mortality of cancer; therefore, it is important to identify which patients may be at higher risk than others, especially before starting specific antitumor treatment. The clinical relevance of TG for predicting cancer-associated VTE is under investigation. Two large clinical observational studies in cancer patients are currently evaluating the predictive value of TG for VTE, as performed with the CAT method.[10] In the first of these studies, the Vienna Cancer and Thrombosis Study (CATS), high values of peak of TG in patients with different types of cancer were independently associated with an increased risk to develop VTE. In the second, the Italian Multicenter HYPERCAN study, preliminary data identified ETP of TG measured before starting first chemotherapy regimen, as an independent predictor of VTE in patients with metastatic breast, lung or gastrointestinal cancer. In a small cohort study of 108 non-metastatic cancer patients undergoing chemotherapy, the evaluation of TG by a different assay was capable of predicting first VTE event.[11] In conclusion, TG seems a promising assay for the evaluation of cancer-associated thrombophilic state. Regarding its role as a predictor for VTE in cancer, an effort in the standardization of the method together with the promising results from the large trials, suggest TG as a tool for the identification of patients at high risk of VTE. The introduction of TG, as well as of other coagulation markers, in published risk scoring models might further help the clinicians in the VTE risk stratification for personalized thromboprophylaxis.

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NON-ONCOGENE ADDICTION-PROMOTING PROTEIN KINASES IN HEMATOLOGIC MALIGNANCIES

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Non-oncogene addiction (NOA) is increasingly being recognized as a peculiar hallmark of cancer. However, little is known about the regulation of this aberrant process in blood malignancies. A number of potential molecules could take part in NOA in multiple myeloma, non-Hodgkin lymphomas and acute leukemia. A common denominator is the property whereby cancer cells are rendered much fitter to cope with various cellular stresses, therefore increasing the probability of selecting more aggressive and apoptosis/necrosis resistant clones.

The serine-threonine kinases CK1 and CK2 are a clear example of proteins exploited by blood cancers to propel their growth. These two kinases have in common the regulation of the Wnt, Hedgehog, PI3K and NF-κB signalling pathways as well as by the ability to dampen the activity of the TP53 tumor suppressor. Preclinical and clinical data indicates that CK1 and CK2 help malignant blood tumor, such as acute leukemia, multiple myeloma and lymphoma, to circumvent growth arresting stress-elicited signals. Increasing evidence suggests that targeting these two kinases with small ATP-competitive, selective inhibitors could represent a rational therapeutic strategy to enhance blood tumor cell death upon conventional and novel agents.

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TARGETING THE CXCR4/SDF1 AXIS IN B-CELL LYMPHOPROLIFERATIVE DISORDERS

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Multiple myeloma (MM) and Waldenstrom's Macroglobulinemia (WM) cells are characterized by the surface expression of CXCR4, known as one of the receptor for CXCL12 (SDF1). Indeed, the Literature has clearly demonstrated that the SDF-1-dependent signaling via CXCR4 plays a critical role in modulating the phenotype of both MM and WM cells, including migration and adhesion, as well as homing to the bone marrow (BM).[1-4] In addition, the interaction of MM and WM cells with the surrounding BM milieu may represent a protective environment, responsible for resistance to therapeutic agents. Azab and Coll. have reported on AMD311-mediated CXCR4 inhibition as a strategy for disrupting the MM cell-to-BM cell interaction, favoring MM cell mobilization to the peripheral blood and ultimately leading to enhanced MM cell sensitivity to multiple anti-MM agents. Importantly, CXCR4 neutralization in MM cells increased the proportion of apoptotic circulating cells when used in combination with bortezomib, thus suggesting its role in enhancing chemosensitivity to anti-MM agents.[2]

Similarly, Ngo and Coll. have dissected the role of CXCR4/SDF1 in regulating adhesion and homing of WM cells to BM microenvironment, including BM endothelial cells and BM mesenchymal stromal cells. Authors showed that CXCR4 is functionally active in WM cells, in response to SDF1, leading to the activation of downstream signaling pathways, such as PI3-AKT, MAPK-ERK. Moreover, these studies have shown how CXCR4-neutralization via AMD3100 was responsible for inhibition of WM cell migration, transendothelial migration, and adhesion.[3]

More recently, we have highlighted the functional role of SDF1 in facilitating MM cell homing and engraftment of clonal MM plasma cells, thus resulting in enhanced MM cell dissemination from bone-to-bone, as demonstrated using in vivo models of MM cell dissemination.[4] These studies have also demonstrated how SDF1-neutralization led to inhibited MM progression.[4]

In addition to CXCR4, CXCR7 has been also reported to be activated by SDF1:[5] for instance, CXCR7 is expressed on MM cells, and it has been reported to be involved in modulating trafficking and adhesion of human malignant hematopoietic cells.[5] Taken together, these findings support the importance of targeting SDF1 in order to achieve a dual neutralization of both CXCR4 and CXCR7. CXCR4 and its ligand SDF1 have been also described as positive regulator of tumor cell metastasis, particularly in the context of solid tumors.[6] We have also recently demonstrated that CXCR4 madiates the acquisition of an epithelial-to-mesenchymal transition (EMT)-like phenotype in MM cells; and that a novel monoclonal antibody anti-CXCR4 leads to inhibited MM cell dissemination to distant bone as well as to extramedullary sites, via modulation of the EMT-like signature.[7]

It has been recently shown that about 30% of WM patients present with an activating somatic mutation of the CXCR4 (C1013G/CXCR4).[8] Specifically, the C1013G/CXCR4 variant enhanced WM cells proliferation in vitro, and facilitated WM cell dissemination to extramedullary organs, leading to disease progression and decreased survival, as shown using animal models.[8] The use of a monoclonal antibody anti-CXCR4 led to inhibition of WM tumor growth, even in the context of C1013G/CXCR4 mutated WM cells.[9] The anti-CXCR4 antibody inhibited p-Akt, p-ERK and p-Src in WM cells cultured in the context of BM stromal cells, and induced apoptosis in a caspase-dependent and –independent manner. The anti-tumor activity driven by the monoclonal antibody anti-CXCR4 was demonstrated both *in vitro* an *in vivo*.[8]

Clinical trial have been initiated for patients with relapsed/refractory MM, testing either CXCR4- or SDF1-neutralizing compounds. Ulocuplumab (anti-CXCR4)[9] and olaptesed pegol (anti-SDF1),[10] used either alone or in combination of other anti-MM agents (lenalidomide; dexamethasone; bortezomib) are being tested for the treatment of relapsed/refractory MM disease. Specifically a phase Ib study has demonstrated that Ulocuplumab-dependent CXCR4 blockade is safe and shows a high response rate of over 50% in relapsed/refractory MM patients, when used in combination with lenalidomde and dexamethasone.[9] In parallel, olaptesed pegol-dependent SDF1-neutralization led to MM cell mobilization to the peripheral blood and enhanced chemosensitivity to conventionally used anti-MM drugs. In particular, olaptesed pegol in combination with bortezomib and dexamethasone resulted in an overall response rate of 68%. Response rates and PFS were similar in patients with or without high risk cytogenetic features or with or without previous exposure to bortezomib. The tested combinatory regimen was safe and well tolerated.[10] Overall, these findings confirm the crucial role of the CXCR4-SDF1 axis in the pathogenesis of B-cell malignancies, including both MM and WM; and provide the preclinical rational for using anti-CXCR4 and/or anti-SDF1 therapies for the treatment of MM and WM.

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TARGET DRUGS IN CHRONIC MYELOPROLIFERATIVE NEOPLASMS

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Traditionally, the therapeutic armamentarium of chronic myeloproliferative neoplasms (MPN) was limited to a handful of cytotoxic agents, including hydroxyurea, busulfan, pipobroman and, at least in some countries, conventional and more recently pegylated interferon-alpha preparations. Following the discovery of *JAK2*V617F mutation in approximately 70% of all MPN patients, and the understanding of an universal involvement of hyperactivated JAK-STAT signaling even in patients with *MPL* or *CALR* mutations, the MPN shyly entered the era of targeted therapy ,[1] inaugurated with the approval of the JAK1/JAK2 inhibitor ruxolitinib in myelofibrosis (MF) first[2,3] and polycythemia vera (PV)[4] more recently. The hope was that we could witness a second "Imatinib-like" era where a single drug produces disease control and, by inducing long-lasting deep molecular remissions in the vast majority of patients, might be associated with the concept of a "cure".[5] Unfortunately, this was not the case for a number of reasons, including the mutational complexity underlying MPN as compared to a monogenic (in a rather simplistic reasoning) disease as is chronic myelogenous leukemia and the non-specificity of ruxolitinib for the mutant JAK2 as compared to the high selectivity of imatinib (and other TKIs) for BCR/ABL. On the other hand, this allowed to obtain similar clinical efficacy in MF patients largely independent of underlying driver, and also subclonal, mutations.[6,7] Although evidence has been provided that with long-term treatment with ruxolitinib some patients may attain even deep molecular responses,[8,9] overall reductions of *JAK2*V617F allele burden are in the range of 20-40%, and the ultimate significance of similar changes for long-term prognosis and/or major disease manifestations is questionable at best. Similarly, some patients may have improvement of bone marrow fibrosis under ruxolitinib, but again this is far from being evidence of morphological remissions. On the other hand, even considering that survival was not a statistically controlled end point of COMFORT studies, owing also the crossover design, there is continuing evidence of a survival advantage in patients who were treated with the JAK2 inhibitor.[10] If referring to PV patients, a part for the optimized control of hematocrit and improvement in symptoms, a signal of reduced cardiovascular events might point to some mechanistic mode of action of ruxolitinib, that wants further exploration.[11] Therefore, while there is no doubt that introduction of ruxolitinib has translated in clinical benefits for most patients, the search for novel key mechanisms/targets is still open and actively pursued. The list of active studies include novel JAK2 inhibitors, inhibitors of the histone deacethylases, of the hedgehog and PI3K/Akt pathways, inhibitors of telomerase, or aurora kinases. These agents are being tested either as single drugs or in combination with ruxolitinib. Finally, although the cell and/or molecular target remain unknown, also interferons are undergoing clinical trials.

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BEST ABSTRACTS

B001

THE USE OF MONOCLONAL ANTIBODY DIRECTED CHIMERIC ANTIGEN RECEPTORS TO FACILITATE TREG CONTROL OF GVHD AND TISSUE TOLERANCE IN MURINE MODELS

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Introduction: GvHD is a severe complication of hematopoietic cell transplantation. Regulatory T cells (Treg) proved to be effective in preventing GvHD in preclinical and clinical studies. Limitations of this approach are Treg paucity and their reduced efficacy when they traffic to inflammation sites.

Methods: We used new technologies of T cell engineering to force the expression of a chimeric antigen receptor on T cells and Treg that recognizes therapeutic monoclonal antibodies (mabCAR), allowing for precise control of their localization *in vivo*. mabCAR expresses a ScFV that recognizes FITC fused to CD28 and TCR costimulatory domains. Any monoclonal antibody (mab) coupled to FITC within its Fc domain can be recognized. We tested this approach with T cells and Treg to ameliorate GvHD and induce tolerance to pancreatic islet grafts.

Results: mabCAR transfected conventional T cells (Tcon) expressed higher levels of CD44 (p=0.0003), CD25 (p=0.009) and produced more IFN (p=0.04) after culture with FITC-mab proving that mabCAR binding drives cell activation. To test if mabCAR transfection alters Tcon and Treg homing after transfer, we injected luc+ mabCAR Tcon directed against MAdCAM1 (a gut and lymph node endothelial integrin) or SDF1 (a chemokine mainly expressed in the bone marrow) into allogeneic hosts. MAdCAM1-directed Tcon mainly homed in gut and lymph nodes, while SDF1-directed Tcon homed to bones and spleen. SDF1-directed Tcon induced a milder GvHD (p<0.001), demonstrating that cell homing impacts GvHD severity. We then tested mabCAR Treg ability to induce GvHD protection. We injected MAdCAM1 directed Treg in an allogeneic GvHD model. mabCAR Treg prolonged survival (p=0.03) and improved GvHD score (p<0.001), thus proving that mabCAR Treg retain regulatory functions. Finally, we tested if mabCAR Treg could induce tolerance to allogeneic pancreatic islet grafts in sublethally irradiated hosts. luc+gfp+ mabCAR Treg homed and expanded over time (p<0.05) on the site of allogeneic islet grafts (right kidney capsule) if incubated with FITC-anti-allogeneic MHC-I mab (see figure). We could detect higher insulin levels in mice that received islets + allo-MHC-I directed Treg in comparison to islets alone (p<0.05) or to islets + isotype-mabCAR Treg (p<0.05) proving that allo-MHC-I directed Treg improved function of allogeneic islets grafts. Allo-MHC-I directed Treg were highly activated (increased CD25, and CD69, p<0.05) and proliferated in the presence of cells that expressed the selected allogeneic MHC-I antigen, but not against host or third-party stimulators proving that mabCAR expression provided Treg functional specificity.

Conclusions: mabCAR expression can be used to control immune cell homing after transfer in different models according to localizing mab availability. We believe that the mabCAR approach may represent a new tool for optimizing cellular therapies to modulate GvHD and for inducing tolerance in organ transplantation.

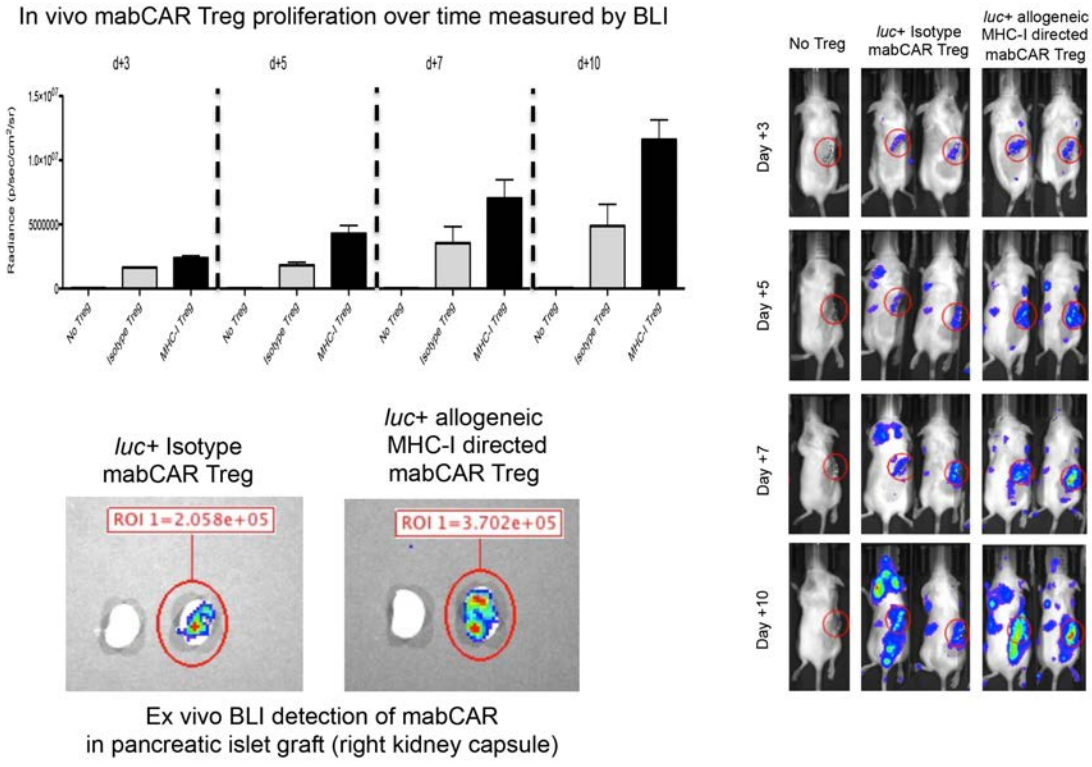

Figure 1.

B002

DEPENDENCE ON GLUTAMINE ADDICTION AND UPTAKE BY MYELOMA CELLS CHARACTERIZES MULTIPLE MYELOMA PATIENTS AND DELINEATES A NEW ATTRACTIVE THERAPEUTIC TARGET

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Introduction: The importance of glutamine (Gln) metabolism in multiple myeloma (MM) cells and its potential role as a therapeutic target are still unknown, although it has been reported that human myeloma cell lines (HMCL)s are highly sensitive to Gln depletion.

Methods: A total cohort of 65 patients with plasma cell (PC) disorders were included in the study: 7 patients with monoclonal gammopathy of undetermined significance, 12 with smoldering myeloma and 46 with active MM. The HMCLs KMS-12-BM, JJN3, RPMI 8226, XG-1 and OPM2 were also used in this study. Ammonium, Gln and glutamate (Glu) levels in the conditioned media of MM cells and in the patient bone marrow (BM) plasma were assessed. The expression of the main enzymes involved in Gln metabolism (Glutaminase (GLS); Glutamine Synthetase (GS) and Asparaginase) and Gln transporters was evaluated in MM cells by Real time PCR and western blot. Cell viability in the presence of Gln enzyme and transporter inhibitors including L-asparaginase, BPTES and CB-839 with or without Bortezomib was assessed by resazurin assay. Furthermore, gene expression profiles of glutamine transporters were evaluated in two PC dyscrasia datasets, generated using 3 proprietary (GSE13591, GSE6205 and GSE66293) and 3 publicly available (GSE6477, GSE6691 and GSE47552) datasets. Finally, anti-SLC1A5 shRNA lentiviral vector was used for ASCT2 stable knockdown in HMCLs, whereas the scramble lentiviral vector was used as control. 8 SCID-NOD mice for group were injected subcutaneously with JJN3 stably transfected with anti-SLC1A5 containing plasmid vectors or with JJN3 stably transfected with the empty vector and then tumor growth was checked.

Results: We found that both HMCLs and primary BM CD138+ cells produced large amounts of ammonium in the presence of Gln. MM patients have lower BM plasma Gln with higher ammonium and Glu than patients with indolent monoclonal gammopathies. Interestingly, HMCLs expressed GLS and were sensitive to its inhibition, while exhibited negligible expression of GS. High GLS and low GS expression were also observed in primary $CD138^+$ cells. Gln-free incubation or treatment with the glutaminolytic enzyme L-Asparaginase depleted the cell contents of Gln, Glu and the anaplerotic substrate 2-oxoglutarate, inhibiting MM cell growth. Consistent with the dependence of MM cells on extracellular Gln, the gene expression profile analysis showed an increased expression of the Gln transporters SNAT1, ASCT2, and LAT1 by $CD138^+$ cells across the progression of monoclonal gammopathies. Among these transporters, only ASCT2 inhibition in HMCLs caused a marked decrease in Gln uptake and a significant fall in cell growth. Consistently, ASCT2 stable down-regulation by a lentiviral approach inhibited HMCL growth *in vitro* and in a murine model.

Conclusions: MM cells strictly depend upon extracellular Gln and show features of Gln addiction. Therefore, the inhibition of Gln uptake is a new attractive therapeutic strategy for MM.

B003

HUMAN APLASTIC ANEMIA DERIVED MESENCHYMAL STROMAL CELLS FORM FUNCTIONAL HEMATOPOIETIC STEM CELL NICHE *IN VIVO*

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Introduction: Aplastic anemia (AA) is a heterogeneous bone marrow failure disorder that in most cases responds to immunosuppressive treatments. Although this suggests an underlying immune mediated process as the main cause of the disease, a number of *in vitro* studies have reported abnormalities in mesenchymal progenitor cell function, thus hinting at a dysfunctional hematopoietic niche. To overcome the limitations of *in vitro* approaches, we have assessed the ability of AA-derived-MSC to form bone and provide hemopoietic niche function using our recently described *in vivo* model based on implant of chondroid pellets in immunodeficient mice.

Methods: We isolated MSC from 8 newly diagnosed AA pediatric patients (AA-MSC) and 7 age-matched healthy donors (HD-MSC) and evaluated their clonogenicity, proliferation, immunophenotype, and differentiation potential *in vitro*. Additionally, we analyzed immunohistologically and functionally the hematopoietic and stromal compartment of the ossicles obtained after the *in vivo* implant of chondroid pellets derived from MSC of both groups.

Results: AA-MSC displayed morphology, phenotypic profile, proliferation and *in vitro* differentiation capacities similar to their normal counterparts, albeit exhibited a lower colony forming efficiency. Moreover, we showed that AA-MSC maintain the capacity to produce *in vivo* ossicles constituted by cortical bone filled with host-derived hematopoiesis. Immunohistochemistry analysis of the hematopoietic tissue in the intertrabecular space within the ossicles revealed the presence of murine macrophages, myeloid cells, megakaryocytes, red blood cells and osteoclasts in similar proportion in normal and patient-derived sections. Moreover, within the AA ossicle-derived marrows, it was possible to determine the presence of hematopoietic clonogenic progenitors in the same number as their normal counterparts. Also the human stromal compartment of the ossicles originated from AA-MSC and HD-MSC resulted similar. Ossicles derived from both sources similarly presented osteoblasts lining the osseous trabeculae, bone marrow interstitial fibrosis, reticulin deposition, iron storages, and contained a comparable amount and disposition of adipose marrow.

Conclusions: This work demonstrates that AA-derived-MSC can form normal hemopoietic niches. Using our *in vivo* model, we have been able to demonstrate that AA-MSC generate ossicles characterized by cortical bone, marrow cavity, donor-derived marrow stroma, and host-derived hematopoietic tissue. Further studies are needed to expand this investigation to a larger cohort of patients, including in particular non-responder to immunosuppressive treatment in which the causative role of the hematopoietic microenvironment in the disease pathogenesis could be more important and functional abnormalities could be identified.

B004

THE CODING GENOME OF CLASSICAL HODGKIN LYMPHOMA (CHL) AS REVEALED BY WHOLE-EXOME SEQUENCING (WES) OF MICRODISSECTED TUMOR CELLS

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Introduction: The genetics of cHL is difficult to study due to the rarity of tumor cells (the Hodgkin and Reed/Sternberg - HRS - cells) in the lymph node.

Methods: We microdissected 1200-1800 single HRS cells per case from frozen biopsies of 36 patients, along with a similar number of non-neoplastic cells, and performed in duplicate whole-genome DNA amplification (WGA) and independent WES sequencing to control for WGA-induced biases. Somatic protein-changing mutations were then validated by deeper sequencing of the same WGA DNA, as well as by ultra-deep sequencing and digital PCR of unamplified whole-biopsy DNA.

Results: We found highly recurrent (31%; 11/36 cases) heterozygous missense mutations in the STAT6 DNA binding domain, identical to the activating alleles known in other B-cell lymphomas. Functional experiments in cHL cell lines showed that STAT6 mutation confers a survival advantage distinct from (and beyond that of) STAT6 phosphorylation (pSTAT6), possibly due to aberrant DNA binding activity following pSTAT6-dependent nuclear entry. Of note, disruptive mutations of the JAK-STAT inhibitor SOCS1 were more frequent in the evaluable STAT6-mutated cases (87%, 6/7 patients) than in unmutated ones (29%, 4/14 patients; p=0.02). This genetic interaction was also validated functionally through wild-type SOCS1 transduction experiments in cHL cell lines. Moreover, the presence in additional cases of genetic lesions targeting other JAK-STAT pathway members (mutations activating STAT3, STA5B, JAK1 or inactivating the inhibitor PTPN1; JAK2 copy number gains) attests to pervasive role of JAK-STAT signaling in the genetics of cHL (up to ~70% of patients affected). We also frequently observed GNA13 mutations (228/36 cases; 22%), similar to the inactivating variants found in other germinal center B (GCB) cell-derived lymphomas. GNA13 ensures the confinement of proliferating GCB cells within lymphoid follicles and at the same time constrains their expansion by facilitating apoptosis in this potentially dangerous niche (Nature 2014;516:254). Thus, mutational disruption of GNA13 may facilitate cHL lymphomagenesis by rescuing from apoptosis crippled GC B cells (the proposed HRS cell precursors) and may promote their dissemination outside the follicle, reminiscent of the interfollicular HRS cell pattern in lymph nodes with partial cHL involvement. Finally, heterozygous missense mutations of XPO1 occurred in 4/36 cases (11%) at the hot-spot E571, recurrently targeted also in other B-cell neoplasms. XPO1 shuttles outside the nucleus >200 proteins, including tumor suppressors involved in cHL pathogenesis and operating in the nucleus (e.g., NFKBIA, FOXOs, TP53). XPO1 is overexpressed in various cancers and its inhibition appears more toxic to tumor than normal cells (Leukemia 2016;30:190).

Conclusions: Our global characterization of the cHL coding genome, the first one based on microdissected tumor cells from a large number of patients, uncovered new genes likely playing an important pathogenetic role in this lymphoma.

B005

CRLF2 OVER-EXPRESSION IS A POOR PROGNOSTIC MARKER IN CHILDREN WITH HIGH RISK T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Although introduction of risk-adapted therapy improved their prognosis, pediatric T acute lymphoblastic leukemia (T-ALL) patients still have a worse outcome compared to B-Cell Precursor (BCP)-ALL patients, with 25% of T-ALL patients with little or no expectancy of cure. They could greatly benefit from the identification of new prognostic markers and potential therapeutic targets. Alteration of Cytokine Receptor-like Factor 2 (CRLF2) gene, a hallmark correlated with poor outcome in BCP-ALL, has not been reported in T-ALL. However, aberrations in IL7R , that heterodimerizes with CRLF2 to form the receptor for thymic stromal lymphopoietin (TSLP), have been described. This observation prompted us to investigate if CRLF2 could also be affected in T-ALL and have a prognostic impact in this pathology too. We analyzed CRLF2 expression in 212 T-ALL pediatric patients enrolled in the AIEOP-BFM ALL 2000 study in Italian (AIEOP) and German (BFM-G) centers. Seventeen out of 120 (14.2%) AIEOP patients presented CRLF2 mRNA expression 5 times higher than the median (CRLF2-high); they had a significantly inferior event-free survival (EFS) (41.2%±11.9 vs. 68.9%±4.6, p=0.006) and overall survival (47.1%±12.1 vs. 73.8%±4.3, p=0.009) and an increased cumulative incidence of relapse/resistance (CIR) (52.9%±12.1 vs. 26.2%±4.3, Hazard ratio=2.84, p=0.007) compared to CRLF2-low patients. The prognostic value of CRLF2 over-expression was validated in the BFM-G cohort. Cox model analysis showed that patients with CRLF2-high expression had a 2.5-fold increased risk of relapse. Interestingly, CRLF2 over-expression was associated with poor prognosis in the high risk (HR) subgroup where CRLF2-high patients were more frequently allocated. Interestingly, although in T-ALL the CRLF2 protein was localized mainly in the cytoplasm, in CRLF2-high blasts we found a trend towards a stronger TSLP-induced pSTAT5 response, sensitive to the JAK inhibitor Ruxolitinib. Moreover, gene set enrichment analysis showed an inverse correlation between the expression of CRLF2 and of cell cycle regulators.

In conclusion, CRLF2 over-expression is a poor prognostic marker identifying a subset of HR T-ALL patients that could benefit from alternative therapy, potentially targeting CRLF2 pathway.

ORAL COMMUNICATIONS

Acute Leukemia 1

C001

LA1 NPM1-MUTATED-SPECIFIC T-CELL RESPONSES OCCURRING IN PATIENTS WITH NPM1-MUTATED ACUTE MYELOID LEUKEMIA

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Introduction: Peptides derived from NPM1mut protein, a leukemia-specific antigen, may elicit *in vitro* specific immune responses by $CD8^+$ and $CD4^+$ T cells, obtained from PB of either patients with NPM1mut AML or healthy subjects, leading to antigen-specific lysis of blasts, as documented by cytotoxicity assays, and potentially contributing to favorable clinical outcome (Greiner et al, Blood 2012;120:1282-9).

Methods: We carried out immunological studies to investigate the occurrence of NPM1mut-specific T cells in 95 BM and 93 PB samples collected, at different timepoints, from 29 adults (median age 56 ys, range 19-75) with NPM1mut AML, using, as antigenic stimulation, either mixtures of 17 short and long peptides (9-18mers) or single peptides, derived from the complete spanning of C-terminal of NPM1mut protein. Five and 14 patients received either allo- or auto-HSCT, respectively. At a median follow-up of 16 months (range 2-91), 10 patients experienced relapse and 21 patients are still alive.

Results: After stimulation with a mixture of all 17 peptides, ELISPOT assay documented NPM1mut-specific T cells producing IFNγ in 45/46 (97.8%) BM samples (median 170 SFC/10^6 cells, range 8-860) and 22/26 (84.6%) PB samples (median 157 SFC/10^6 cells, range 22-736), irrespective of HLA-restriction. Subsequent analyses identified LAVEEVSLR and AVEEVSLRK (peptides 13-14) as the most immunogenic 9mer peptides derived from NPM1mut protein. NPM1mut-specific T cells producing IFNγ were shown by ELISPOT assay after stimulation with peptides 13-14 in 41/49 (83.7%) BM samples (median 83 SFC/10^6 cells, range 6-546) and 55/67 (82.1%) PB samples (median 147 SFC/10^6 cells, range 6-696). Of interest, IFNγ-producing specific immune responses were early observed in all available samples obtained from 13 patients after induction chemotherapy. MRD monitoring for NPM1mut by RQ-PCR have been performed on 86 BM samples from 18 patients. In 12 subjects experiencing long-term morphologic CR for whom samples were available, MRD was undetectable. Interestingly, high frequencies of IFNγ-producing NPM1mut specific T cells were commonly found in either BM or PB samples collected from 14 patients more than 12 months after AML diagnosis. Furthermore, cytokine production and memory T-cell profiles, analyzed by multiparametric flow cytometry in 26 BM samples from 14 patients, showed the presence of NPM1mut-specific T cells mainly producing IFNγ, with lower levels of IL2- and TNFα-secreting cells. Both Effector Memory and Central Memory T-cell phenotypes, mainly $CD8^+$ and $CD4^+$, respectively, were observed. Of interest, a subset of CD107+ cytotoxic NPM1mut-specific T cells was identified.

Conclusions: We observed the spontaneous development of NPM1mut-specific T cells in BM and PB of NPM1mut AML patients, which may contribute in maintaining long-lasting remissions. Further studies are warranted to investigate the dynamics of such specific immune response and a potential role of immunotherapeutic approaches in NPM1mut AML.

C002

PRDM16 OVEREXPRESSION IS RECURRENT IN ADULT AML PATIENTS AND CORRELATES WITH SPECIFIC GENETIC AND CLINICAL FEATURES

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Introduction: PRDM16 overexpression has been recently reported in paediatric AML with high-intermediate risk cytogenetic abnormalities, FLT3-ITD and NUP98-NSD1 rearrangement and it has been independently associated with an adverse outcome. In adult AML, PRDM16 overexpression has been reported as consequence of rearrangements involving 1p36 region, while few studies have investigated PRDM16 expression in absence of 1p36 abnormalities by conventional cytogenetic (CC). The aims of our study were: 1) to identify PRDM16 involvement in AML/MDS with 1p36 abnormalities; 2) to assess PRDM16 expression in high-intermediate risk cytogenetic AML.

Methods: Twenty-six AML/MDS with 1p36 abnormalities and 113 AML without 1p36 involvement by CC were analyzed by relative RQ-PCR and FISH analysis using 3 BAC probes for PRDM16. RESULTS: FISH analysis identified 13/26 (50%) cases with PRDM16 abnormalities. The most common one was t(1;3)(p36;q21) (n=6), other 4 cases showed unique translocations among which t(1;10)(p36;q12) and t(1;17)(p36;q23), were never reported before. Patients were young (median age 56) and were diagnosed mainly as MDS or secondary AML. PRDM16 rearrangements were not associated with complex karyotype and the most common additional abnormality was del(5q). The other 3 cases showed copy number gain (CNG) of PRDM16; they were de novo AML and were associated with complex karyotype. Both rearrangement and CNG were associated with PRDM16 overexpression. After median follow-up of 16 months, only 4 patients were alive: 3 underwent to alloSCT and were in complete remission. All cases with PRDM16 abnormalities were FLT3 and NPM1 wild-type. High levels of PRDM16 expression (PRDM16-high) were observed in 39 AMLs without 1p36 abnormalities: 17 cases were cytogenetically normal (CN), 9 had complex karyotype, 6 cases had isolated -7/7q-, 5 had rare isolated cytogenetic abnormalities and 2 had inv(3)/t(3;3). By FISH, no cryptic PRDM16 rearrangements were identified, only 3 cases with complex karyotype showed an extra copy of PRDM16. In CN-AML, FLT3-ITD were more frequent in PRDM16-high cases than in cases with low levels of PRDM16 expression (PRDM16-low) (48% vs 14%; p=0.01); whereas no statistical difference was observed in the frequency of NPM1 mutations (48% vs 41%; p=0.78). No difference was observed in overall survival between PRDM16-high and PRDM16-low patients, however, PRDM16-high patients showed high rate of relapse (50% vs 0%;p=0.03).

Conclusions: PRDM16 was frequently overexpressed in cases with 1p36 abnormalities either due to rearrangements or CNG involving PRDM16. PRDM16-rearranged cases showed specific features such as a young age at diagnosis, non-complex karyotype,

absence of NPM1 or FLT3 mutations and a poor outcome. PRDM16 overexpression, even without PRDM16 abnormalities, was found in a subset of AML with high-intermediate risk cytogenetic abnormalities. In CN-AML, PRDM16 overexpression was associated with FLT3-ITD and high rate of relapse.

C003

INNOVATIVE, CAPTURE-BASED NEXT GENERATION SEQUENCING (NGS) APPROACH FOR IG/TCR CLONAL MARKERS IDENTIFICATION IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS AT DIAGNOSIS

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Background: Minimal Residual Disease (MRD) is the most important prognostic factor in Acute Lymphoblastic Leukemia (ALL). To date, clonal markers for MRD monitoring are identified at diagnosis by PCR amplification of the most frequent IG/TCR rearrangements. Unfortunately, in a proportion of cases this approach fails. Recently, alternative Next Generation Sequencing (NGS) approaches for clonality assessment and MRD monitoring have been described. Despite the high sensitivity, these methods are similarly based on commonly used rearrangements amplification and, therefore, can suffer the same limitation of conventional method in identifying rare Ig/TCR rearrangements.

Aims: To identify IG/TCR clonal markers at diagnosis by a novel capture-based NGS approach, possibly overcoming limits of an amplicon-based approach.

Methods: We designed a capture-based NGS panel on V, D and J genes in the IG/TCR loci. Libraries were prepared by Nextera Rapid Capture Enrichment protocol (Illumina) and pair-end sequenced on the MiSeq platform. To validate this NGS approach, we studied 10 diagnostic bone marrow samples of adult ALL patients formerly characterized for clonality within the NILG09/00 trial (ClinicalTrials.govId:NCT02067143) following the EuroMRD guidelines. As negative controls we included 2 Human Umbilical Vein Endothelial Cells (HUVEC) and 2 mesenchimal cord blood cells. NGS data were analyzed by the Vidjil software (http://www.vidjil.org).

Results: In the 10 diagnostic samples, capture NGS confirmed the presence of 50 clonal rearrangements previously identified by standard method. Only one IGH rearrangement was not confirmed because of low coverage in the involved V, D and J genes. Moreover, our approach identified 24 additional clonalities that were later confirmed: (a) 11 were characterized by uncommon V/DJ combinations amplified and sequenced only after NGS based oligonucleotide design; (b) 10 were oligoclonal rearrangements in which Sanger did not allow the discrimination of single sequences that was only possible by NGS; (c) 3 were low represented clones that were revealed only after reamplification and sequencing of faint heteroduplex amplicons. No rearrangement in IG/TCR genes of HUVEC and mesenchimal cells was revealed by capture NGS data analysis.

Conclusions: Our capture-based NGS approach for IG/TCR allowed to identify uncommon and low represented rearrangements not isolated by conventional methods. Capture NGS can be of high value in patients in which standard procedures could not identify clonal markers for MRD evaluation, allowing them to benefit from an MRD driven treatment. This technology also offers the opportunity to identify minor leukemia clones at diagnosis so preventing unexpected relapse in patients negative for major clones. In the future, we will apply this approach where standard clonality studies failed so to redefine MRD risk classification and to correlate it with clinical outcome within the NILG trials.

C004

ANTI-LEUKEMIC EFFICACY OF BET INHIBITOR TREATMENT IN A PRECLINICAL MODEL OF INFANT MLL-AF4+ ACUTE LYMPHOBLASTIC LEUKEMIA

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Despite the improvements in the field of pediatric leukemia, some groups of patients still suffer from a low cure rate. MLL-rearranged Acute lymphoblastic leukemia occurring in infants (MLL+ infant ALL) has an overall poor outcome. Numerous studies have shown that the perturbation of the chromatin structure and the transcriptional deregulation induced by the MLL fusion represent the main mechanism of leukemogenesis. Therefore, the use of epigenetic compouds targeting the chromatin structure and/or the recruitment of the transcriptional complex represents nowadays a promising approach.

In this study we aimed to investigate the anti-leukemic effect of Bromodomain and Extra Terminal (BET) inhibition by using the I-BET151 inhibitor. BET is a family of adaptor proteins (BRD2, BRD3 and BRD4), binding to the acetylated chromatin.

The anti-leukemic effects of I-BET151 administration was tested *in vivo* in a preclinical model of human MLL$^+$ infant ALL. A human MLL$^+$ cell line or primary samples derived from infant patients were transplanted into immunedeficient mice, in order to recapitulate the disease *in vivo*. Subsequently, the animals were treated i.p. with I-BET151 or with the vehicle only. Additionally, the biological mechanism of the compound was further elucidated at the molecular lever, both *in vitro* using several human MLL$^+$ ALL cell lines, as well as ex vivo in xenograft samples from transplanted mice.

We observed that I-BET151 administration reduces the engraftment and the disease burden of MLL+ infant ALL *in vivo* and prolongs the survival in mice. Importantly, the anti-leukemic effect of I-BET151 was also confirmed in a "curative setting" experiment, where the treatment was started when the leukemia was already consolidated in mice. Furthermore we elucidated the biological mechanism of the compound and demonstrated that I-BET151 is able to block cell proliferation (G0/G1-phase arrest) and induce apoptosis through the downregulation of known BRD4 target genes (i.e. c-MYC, BCL2 and CDK6) and the impairment of the IL7Ra/STAT5 signaling pathway. Moreover, the analysis of transcriptional profile changes of PDX samples exposed to I-BET151ex vivo allowed us to identify a specific "I-BET core signature" of genes deregulated upon treatment and belonging to the BRD4 and HOXA gene networks. The function of these newly identified genes and their potential role in MLL leukemogenesis is currently under investigation. Finally, we have observed that I-BET151 in combination with HDAC inhibitors is even more efficient compared to the single therapy, as these two compounds have a synergic activity. Our data show that I-BET151, alone or in combination with HDAC inhibitors, exerts a potent anti-leukemic effect in MLL+ infant ALL. In conclusion, given the aggressiveness of the disease and the lack of a cure for infant patients with MLL leukemia, this study is particularly relevant, as I-BET151 may represent a promising novel approach for future therapeutic interventions.

C005

TCF7/5Q31 DELETION MARKS A HIGHLY AGGRESSIVE EARLY T-CELL PRECURSOR LEUKEMIA (ETP-ALL) SUBGROUP

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Background. T-ALL is characterized by co-occurrence of multiple lesions which perturb specific cell processes and drive development of overt leukemia. Type A lesions cause oncogene over-expression and define distinct genetic entities. Type B, co-operate with type A lesions and interact each other. As they are mainly represented by loss-of-function mutations and/or deletions, oncosuppressor genes likely play a pivotal role in T-ALL pathogenesis. We aimed to get insight into del(5q) in T-ALL.

Methods. We studied del(5q) in a cohort of 290 T-ALL patients enrolled into the GIMEMA, AIEOP, and MRC, UK, clinical trials. Del(5q) were investigated by FISH with probes for rearrangements of TLX3, NPM1, and sub-telomeric 5q sequences. Cases with del(5q) were further characterized with 26 clones for 5p13-qter and SNPa. CI-FISH to investigate 51 genes/loci, identified T-ALL associated genomic rearrangements and classified cases into distinct genetic categories. DHPLC, Sanger sequencing, Haloplex PCR, trascriptome sequencing, and GEP were performed.

Results. Overall del(5q) were detected in 29/290 cases. According to the extension of deletion, a common region of loss (CDR) at 5q31, was detected in 20 cases; 6 with interstitial and 14 with terminal 5q deletion. This CDR encompassed the putative onco-suppressor gene TCF7 which was significantly down-regulated indicating that deletion caused gene haplo-insufficiency. TCF7 deletion (TCF7-/+) was significantly associated with female gender (M/F ratio=0,6; chi-square, p=0,004) and adult age (p=0,023), and clustered into the HOXA category (14/20) (p<0,001). Within the HOXA category, TCF7-/+ marked a distinct subgroup of T-ALL characterized by an early stage of blast differentiation (9/14 cases; 6 fulfilling ETP diagnostic criteria and 3 pre-T ALL expressing myeloid markers) and/or a genetic profile closely resembling ETP-ALL. Namely, high rate of JAK1/3, ETV6, WT1, RB1, DNM2, RUNX1, PHF6 involvement as well as a significantly low rate of CDKN2AB deletion (p<0,001) were found. In addition to TCF7, also TDRKH, PCGF5, HDAC4, and MTA3 were significantly down-regulated so that, this leukemia subgroup, carried 5/10 top down-regulated genes seen in ETP-ALL. Finally, a significantly high expression level of MEF2C, LMO2, and CD34, typically up-regulated in ETP-ALL, was observed. Other HOXA positive TCF7-/+ ETP-ALL associated abnormalities were del(6q) and aberrations of the TP53/ATM pathway. Although it needs to be confirmed in largest patient cohorts, HOXA positive ETP-ALL with TCF7-/+ appeared associated with a particularly dismal outcome (38% vs 59% of long term survivors).

Conclusions. Deletions of the long arm of chromosome 5 are a recurrent cytogenetic abnormality in T-cell acute lymphoblastic leukemia, occurring in ~10% of cases. Our data provided evidence that TCF7-/+ marks a highly aggressive cytogenetic subgroup within the HOXA positive ETP-ALL category for which, alternative therapeutic options, should be considered.

C006

TP53 GENE MUTATIONS DO NOT AFFECT THE HEMATOLOGIC RESPONSE ACHIEVEMENT BUT CORRELATE WITH EARLY RELAPSE AND VERY POOR OUTCOME IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction and Aim: Although most adults affected by Acute Lymphoblastic Leukemia (ALL) enter complete hematologic remission after induction therapy, only 30-40% survive 5 or more years. Conventional risk factors, immunophenotype and cytogenetic remain valid but insufficient tools at diagnosis to define prognosis. Minimal Residual Disease (MRD) during treatment is the most important prognostic factor for outcome in patients affected by ALL, but it does not represent a stratification risk factor at diagnosis. Recently, few studies on heterogeneously treated patients reported that TP53 mutations correlate with poor response to induction therapy and short survival. We evaluated the impact of TP53 alterations on the outcome of 171 Philadelphia-negative adult ALL patients with a long follow-up (up to 15 years) enrolled into the NILG-ALL 09/2000 multicenter clinical trial (ClinTrials.gov identifier: NCT00358072).

Methods: We investigated the presence of TP53 mutations in exons 4-11 by deep sequencing using a GS Junior Platform (Roche Diagnostics, Mannheim, Germany) on a total of 171 DNA diagnostic samples. Moreover, the copy number status of TP53 was evaluated by quantitative PCR method using hTERT as reference gene for 158 patients (Applied Biosystems, Foster City, CA).

Results: Mutations of TP53 occurred in 8% of patients with a higher prevalence in B lineage ALL (9.6 vs. 5.3% in T lineage). These TP53 alterations were classified as missense or frame-shift mutations occurred in the DNA binding domain with a wide mutation load (97-4%) suggesting that TP53 alterations can be present at diagnosis in minor leukemic clones. By univariate analysis, TP53 mutations were strictly related to older age (p=0.0003) and their presence did not impair remission achievement. Nevertheless, relapse rate was significantly higher in mutated cases (p<0.0001). Moreover, TP53 mutated patients showed a dramatically shorter LFS and OS compared to wild-type (p=0.0008 and p=0.0013, respectively). The multivariate analysis confirmed that the presence of a TP53 mutation at diagnosis was independently associated with a worse clinical outcome. The analysis of the TP53 copy number status revealed that 10 out of 158 (6.3%) patients presented one copy of the TP53 gene. Five of these 10 patients belong to the cohort bearing also a TP53 mutation (38.5%), demonstrating a strong correlation between these two genetic alteration (p=0.00049), as previously described. However, the solely TP53 deletion did not show to have any impact on clinical outcome.

Conclusions: Since the presence of TP53 alterations at diagnosis defines a group of patients with a very poor outcome, the definition of the mutational status of this gene must be included in the diagnostic work-up of adult ALL. The broad range of mutation load we

found in our diagnostic specimens underlines the importance of evaluating samples with a highly sensitive technique, as NGS methodologies, also at disease presentation.

C007

DNA/RNA NEXT GENERATION SEQUENCING-TARGETED CAPTURE STRATEGIES FOR THE IDENTIFICATION OF NOVEL FUSION GENES, IN CHILDHOOD B-CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction: Up to 15% of pediatric patients affected by B-Cell Precursor Acute Lymphoblastic Leukemia experience relapse, which is still a leading cause of cancer-related death in children. Thus, a major effort is dedicated to improve diagnosis tools, and identification of new prognostic markers and new targets for personalized therapy. In this context, the objectives of present study are: (i) to set up a Next Generation Sequencing Target-Capture (NGS-TC) panel focused on ALL, capable of identifying both novel and conventional fusion genes of specific target genes; (ii) to provide a user-friendly bioinformatics platform to detect such genomic alterations. Our final aim is to provide a single protocol for routine cancer diagnostics and identification of new targets for personalized therapy.

Methods: Two NGS-TC approaches were evaluated: (i) on DNA, by Nextera Rapid Capture Custom panel (Illumina), 17 targets; (ii) on RNA, by designing and comparing alternative panels: SureSelect (Agilent) and Ovation Fusion Panel Target Enrichment System (Nugen), 19 and 77 targets respectively; in addition, a pre-designed RNA TruSight Pan-Cancer (Illumina) was tested (1385 targets). All the NGS-TC approaches were set on the MiSeq platform (Illumina). All datasets were analyzed for fusion genes by a purpose-built bioinformatics tool. RNA datasets were additionally analyzed by the TopHat Alignment App on the Illumina BaseSpace Cloud.

Results: As positive controls, a subset of previously characterized samples was analyzed by NGS-TC. Conversely, novel fusion genes were detected and validated by RT-PCR. DNA datasets analysis (N=65 patients) detected both novel and known fusion genes, such as PAX5-fusions or ABL1-fusions with several partners (N=5 and N=3, respectively) and EBF1/PDGFRB. On the other hand, a subset of known gene fusions were not detected (e.g. P2RY8/CRLF2), most probably as a consequence of a non-homogenous target coverage which affected all the DNA target capture experiments. RNA datasets analysis (N=91 patients) detected known fusions, such as BCR/ABL1 (N=8), ETV6/RUNX1 (N=12), MLL/AF4 (N=4), TCF3/PBX1 (N=6) and EBF1/PDGFRB (N=1). In contrast to DNA, the RNA-based approach was also successful in detecting P2RY8/CRLF2 cases (N=2). Moreover, novel fusions involving JAK2 (N=2) and TCF3 (N=2) were detected. Finally, 14 samples were analyzed by both DNA and RNA. The three RNA target capture protocols were transversally analyzed to compare library preparation flowcharts in terms of amount of RNA (from 100ng to 2ug), timeline (2 day up to 5 days), man-effort, efficiency and cost.

Conclusions: We identified fusion genes among the Italian cohort of children with BCP-ALL, confirming the frequent involvement of B-cell genes in fusion events. Overall, we conclude that RNA target capture is the most feasible solution to translate NGS-TC from a research tool to a routine diagnostic protocol, being able to detect both conventional and novel fusion genes.

C008

IDENTIFICATION AND CHARACTERIZATION OF NOVEL RARE NUCLEOPHOSMIN (NPM1) GENE MUTATIONS IN ACUTE MYELOID LEUKEMIA (AML) BY A COMBINATORIAL APPROACH OF IMMUNOHISTOCHEMISTRY AND MOLECULAR ANALYSES

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Introduction: Nucleophosmin (NPM1) mutations occur in 50–60% of adult AML with normal karyotype. About 50 NPM1 mutations have been so far identified, all clustering in exon-12 but few sporadic cases involving either exon-9 (one) or exon-11 (two). In spite of molecular heterogeneity, all mutations cause common changes at the C-terminus of NPM mutants, i.e. loss of tryptophans 288 and 290 (or 290 alone) and creation of a new nuclear export signal (NES) motif. As a consequence, all NPM1 mutants aberrantly accumulates in the cytoplasm of leukemic cells and can be detected by immunohistochemistry, which is fully predictive of NPM1 gene mutations.

Methods: From 2005 to 2015, 702 AML patients samples were analyzed at diagnosis by both immunohistochemistry (IHC) for NPM1 subcellular localization and western blot (WB) with anti-NPM1 mutant antibodies. Discordant cases were further analyzed by NPM1 gene Sanger sequencing. Newly discovered NPM1 mutated genes were subcloned in pEGFP-C1 vector and transiently expressed in NIH-3T3 adherent cells to study the NPM1 mutant subcellular localization by immunofluorescence microscopy. The NESbase version 1.0 program was used to identify putative NES within the new protein sequence, and their efficiency was evaluated by the pREV1.4-based NES efficiency assay.

Results: At IHC and WB analyses, concordance in diagnosis was obtained in 695/702 samples (291 NPM1-mutated and 404 NPM1-unmutated AML). In 7/702 (1%), IHC detected cytoplasmic NPM1 whilst WB with anti-NPM1 mutant antibodies was negative. Unfortunately, in 3 out of these cases, the original patient sample was not available for further analyses. In the other 4, exon-12 NPM1 gene sequence was wild-type (WT), in keeping with the negative WB results. One of these cases harbored the previously described exon-11 NPM1 mutation, in 1 case no mutation was detected (further studies are ongoing), and in 2 cases new mutations involving exon-6 were discovered. Strikingly, in the latter cases, WB analysis with different anti-NPM1 antibodies revealed a new band at different molecular weight (MW) than NPM1-WT. Indeed, in 1 case an in frame 21 nucleotides insertion at exon-6 lead to a 7 aa longer than WT protein, whilst in the other a 19 nucleotides insertion created a new stop codon leading to a truncated protein. In both cases, a new NES motif was created. Importantly, cell transfection experiments confirmed that the new NPM1 mutants localized at least partly in the cytoplasm, and the pREV1.4-based NES efficiency assay showed the new NES were active.

Conclusions: Here, we report on the identification and functional characterization of two novel NPM1 mutations in AML. Our observations further support the view that cytoplasmic NPM1 dislocation is a critical step in leukemogenesis, and that immunohistochemistry, that detects, through cytoplasmic dislocation on NPM, 'all types' of NPM1 mutations, might be used as first step for directing further molecular studies.

Monoclonal Gammopathies and Multiple Myeloma 1

C009

MOLECULAR AND CLINICAL TYPES OF PLASMA CELL DYSCRASIAS ARE ASSOCIATED WITH DISTINCT EXPRESSION PATTERNS OF LONG NONCODING RNAS

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Multiple myeloma (MM) is a malignant proliferation of antibody-secreting bone marrow plasma cells (PCs) characterized by a wide clinical spectrum ranging from the presumed pre-malignant condition called monoclonal gammopathy of undetermined significance (MGUS), to smoldering MM (SMM), truly overt and symptomatic MM, and extra-medullary myeloma/plasma cell leukemia (PCL). As the role and significance of long non-coding RNAs (lncRNAs) in PC malignancies remains virtually absent, we developed a custom annotation pipeline on Gene 1.0 ST microarray data, able to investigate 1852 lncRNAs (annotated in LNCipedia v3.1) in highly purified bone marrow PCs from 20 MGUS, 33 SMM, 170 MM, 36 PCL and 9 healthy donors, collected from proprietary and publicly available datasets. Our study identified 31 lncRNAs deregulated in pathological samples compared to the healthy condition. In particular, the upregulation of MALAT1 in symptomatic patients was associated with molecular pathways involving cell cycle regulation, p53-mediated DNA damage response, and mRNA maturation processes. Furthermore, we found 21 lncRNAs whose expression was progressively deregulated trough the more aggressive stages of PC dyscrasia, suggesting a possible role in the progression of the disease. In the context of molecular heterogeneity of MM, we identified a transcriptional fingerprint in hyperdiploid (HD) patients, characterized by the upregulation of lncRNAs/ pseudogenes related to ribosomal protein genes. These findings are in line with the global up-regulation of the translational machinery, including genes involved in protein biosynthesis, characterizing HD group. To gain evidences of lncRNAs that may potentially act on gene expression, we evaluated the correlation of each of the differentially expressed lncRNAs with all the transcripts unambiguously detectable by the arrays. By focusing on high correlation coefficient, we identified five lncRNAs suggestive of an in cis rather than an in trans interaction. Finally, based on several evidences that have suggested the interplay of the various non-coding species, we investigated the liaison between lncRNAs and miRNAs in 125 samples out of our series for which miRNA expression profiling was available. We identified 290 lncRNA-miRNA couples significantly anti-correlated in our database. Hence, to add confidence to the anti-correlation connection, we inspected which of the 290 lncRNAs anti-correlated to miRNAs could also be miRNAs targets. Based on RNA22 prediction algorithm, we identified nine lncRNA-miRNA couples with transcripts resulting anti-correlated and for which lncRNA is a predicted miRNA target. Particularly interesting is lnc-MAP1LC3B2-2, found upregulated in MM with MAF translocation and anti-correlated with miR-222 and miR-221.Our study reported many lncRNAs deregulated in different forms of PC dyscrasia, providing an important source for future functional studies on lncRNAs in MM disease.

C010

IN MULTIPLE MYELOMA THERE ARE TWO SUBSETS OF IMMUNOSUPPRESSIVE POLYMORPHONUCLEAR NEUTROPHILS WITH INCREASED LEVELS OF ARGINASE

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Development of MM is associated with accumulation of Myeloid-Derived suppressor cells (MDSC), mostly represented by pathologically activated relatively immature polymorphonuclear neutrophils (G-MDSC). Our previous work showed that also mature polymorphonuclear neutrophils (PMN) are activated and immunosuppressive in MM. We evaluated neutrophils by dividing them in low-density neutrophils (LDN), isolated by upper Ficoll layer and high-density neutrophils (HDN), sorted from the lower Ficoll layer, isolated from MGUS or MM patients. Using oligonucleotide microarrays we first evaluated the gene expression profile (GEP) of HDN in 10 MM, 5 MGUS and 8 healthy subjects (HS) identifying Arg-1 as the first gene differentially expressed in MM versus healthy PMN. Thus, we validated Arg-1 by RT-PCR, immunohistochemistry. and circulating levels in serum by ELISA in 60 MM patients, 30 MGUS and 30 HS in both G-MDSC and PMN. MM-LDN exhibit an increased expression of ARG-1 compared to MGUS and HS ($p=0.003$), confirmed by functional assay of enzymatic activity of ARG-1, positively correlated with advanced disease. Immunostaining showed that Arg-1 was increased in MM versus HS, but higher levels were evident in LDN than HDN. Moreover, in MM-LDN Arg-1 was evident at the nuclear level, while in HDN Arg-1 had an cytoplasmic localization. This differential distribution of Arg-1 was functionally evident, since LDN were more immunosuppressive than HDN. Indeed, after 72 hours of co-culture with T-cells obtained from healthy donors in presence of mitogen stimulation (PHA), MM-HDN were able to reduce T-cell proliferation at both tested 1:2 and 1:8 ratios ($p<0.0001$), while MGUS-HDN induced a partial inhibition only at the 1:8 ($p=0.002$). This effect was partially reverted with the treatment of 200 μM norNOHA, an Arg-1 inhibitor, since within first 24 hours T-cell proliferation increased in presence of MM-HDN ($p<0.0001$) and MGUS-HDN ($p<0.0001$). MM-LDN were more immunosuppressive than their MM-HDN counterpart, since at 72 hours T-cell proliferation was 22.3 ± 1.6 % and 8.4 ± 0.5 % ($p<0.0001$) at 1:2 and 1.8 ratio respectively. When evaluated by transmission electron microscopy (TEM), HDN appeared as mature neutrophils without ribosomes and endoplasmic reticulum, enriched of secondary grains containing Arg-1, while LDN were enriched of primary grains, with a peculiar nuclear localization of Arg-1.Finally, we found that circulating serum Arg-1 was higher in MM than MGUS and HS ($p=0.0022$). In MM patients there was a progressive increase from ISS stage I through III ($p=0.003$). In addition, increased levels of ARG-1 were positively associated with advanced bone disease ($p=0.02$) and unfavorable cytogenetics ($p=0.022$).Polymorphonuclear neutrophils in MM are immunosuppressive, but distinguished in two main subpopulations, at different stages of maturation, based on the expression of Arg-1 and grains distribution. S-Arg-1 is increased in MM patients and correlated with adverse features.

C011

CK1α INACTIVATION TRIGGERS AUTOPHAGY IN MULTIPLE MYELOMA

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Introduction: Multiple myeloma (MM) is a debilitating malignancy arising from plasma cells in the bone marrow. This disease is still incurable and new therapeutic strategies are urgently needed. The Ser/Thr kinase CK1α regulates many signaling pathways involved in MM pathobiology such as Wnt/β-catenin, HH and PI3K/AKT/mTOR and it was recently demonstrated that it is over-expressed in MM and that it has an oncogenic role in MM, supporting growth, survival and proliferation of malignant plasma cells. Autophagy is an essential process for the survival of both normal and malignant plasma cells and the downmodulation of caspase 10, a protein that protect MM from excessive autophagy, caused MM cells apoptosis. Considering that an unknown role of CK1α in the downmodulation of the autophagic pathway was recently determined in other cancer types, we aimed at investigating whether CK1α inactivation could induced MM cells death through an excess of autophagic activation.

Methods: MM cell lines used were U-266, H929 and the IL6-dependent INA-6. Malignant plasma cells were purified from bone marrow or peripheral blood of MM patients. Protein Kinase CK1α was inhibited with the small ATP-competitive compound D4476 and it was silenced by different means (ds siRNA oligonucleotides or generation of CK1α IPTG inducible MM clones with transduction of lentiviral particles). The effects of CK1 inactivation on autophagy related proteins were investigated by WB and real-time PCR. The levels and localization of the autophagy-related transcription factor FOXO3a was evaluated by nuclei extraction and WB.

Results: CK1α inactivation with D4476 or through RNA interference resulted in MM cells apoptosis. Upon CK1α inactivation, the kinase AKT, which activates the autophagy inhibitory complex mTORC1, was downmodulated and the lipidated form of LC3 protein was increased, thus suggesting an activation of the autophagic process. Moreover, the pro-autophagic transcription factor FOXO3a, phosphorylated by AKT and CK1α, which is normally sequestered in the cytoplasm, accumulated in the nuclei upon CK1α inactivation, indicating that autophagic related genes are transcribed. Finally, the transcription factor IRF4, which controls caspase 10 gene transcription, resulted downregulated upon CK1α inactivation, pointing to a mechanism whereby protection from excessive autophagy is disrupted.

Conclusions: Taken together our results suggest that exuberant autophagic pathway is triggered upon CK1α inactivation. We also found an unexpected downregulation of IRF4 protein that occurred by a mechanism to be clarified. Considering IRF4 importance in the transcription of caspase 10, a protein that in MM discriminated from pro-survival autophagy and excessive autophagy, it conceivable to speculate that CK1α inhibition caused MM cell death through an excess of autophagy.

C012

EXPRESSION AND ROLE OF CD38 IN THE BONE NICHE OF MULTIPLE MYELOMA: POTENTIAL EFFECTS OF DARATUMUMAB

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Introduction: The use of monoclonal antibodies shows promising results in the treatment of multiple myeloma (MM). Recently, Daratumumab (DARA), a human anti-CD38 monoclonal antibody has been developed with broad-spectrum killing activity. Moreover, DARA treatment seems to induce a re-distribution of the CD38 molecules in MM cells and a formation of polar aggregates with a subsequent release of microvesicles (MV). However, DARA effects in the context of MM bone niche and on MM–induced bone disease are unknown. Thus, in this study, we analyzed the expression of CD38 and its related ectoenzymes in bone niche cells and the potential effect of DARA on bone cells.

Methods: CD38, CD73, CD39, CD203, CD157 and CD31 expression was evaluated by immunohistochemistry on bone biopsies of a cohort of 38 patients with MM and 14 with monoclonal gammopathy of uncertain significance (MGUS). The same antigens were analyzed by flow cytometry on primary MM cells and human myeloma cell lines (HMCLs), mesenchymal stromal cells (MSC), osteoblasts (OB), monocytes and osteoclasts (OC). Then, we tested DARA (1-25 ug/mL) effects, as compared to human IgG isotype control, on OB formation, proliferation and survival, and on OC differentiation. OC number and activity were evaluated by both TRAP staining and a fluorimetric osteolysis assay. We also investigated the effect of MV isolated from HMCLs, treated with DARA (200 ug/mL) or the human IgG isotype control, on OC differentiation.

Results: MM cells showed a high expression of CD38 and were positive only for CD203 and for CD39 and CD31, at variable levels. However, we did not find any significant difference in the expression of CD38 and related ectoenzymes between MM and MGUS patients. CD38 was also expressed by BM monocytes but not by OC, BM MSC and OB, that were positive for CD73 and CD203. According to CD38 distribution in BM niche, we further demonstrated that DARA binds both MM cells and monocytes, but not OB and OC. In line with these results, DARA did not show any effect on OB formation, proliferation and survival. Meanwhile, we found that DARA, at concentration higher than 10 ug/ml, significantly inhibited OC formation and activity with a dose dependent effect, from BM mononuclear cells (MNCS) and from the CD138-cell fraction. This effect was observed only with DARA treatment from day 0 of the culture period. On the other hand, late OC progenitors and mature OC were not affected by DARA treatment. This could be explained by the loss of surface CD38 expression by monocytes cultured in a pro-osteoclastogenic medium after 7 days. On the other hand, we did not find any significant effect of DARA-induced MV on OC formation from BM MNCS, compared to MV obtained in the presence of IgG isotype control.

Conclusions: Our data suggest that DARA inhibits osteoclastogenesis, targeting monocytes and early OC progenitors, giving a rational basis for the use of an anti-CD38 based approach as a treatment for MM bone disease.

C013

CHARACTERIZATION OF TRANSCRIPTIONAL PATTERN OF MIRNAS AND MORNAS IN MULTIPLE MYELOMA BY SMALL RNA SEQUENCING

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Introduction: High-throughput analyes of the miRNome of primary multiple myeloma (MM) tumors have unraveled the differential expression of miRNAs in specific cytogenetic subgroups and demonstrated the usefulness of their inclusion in prognostic models. However, the investigation of miRNA expression in MM, albeit extensive, has been limited to date to microarray/PCR-based approaches, without exploiting all the possibilities offered by small RNA-sequencing (RNA-seq). These include improved detection of known/new mature species, accurate quantification of isomiRs and a comprehensive investigation of miRNA-offset RNAs (moRNAs; miRNA-like RNAs produced by non-canonical processing of the pre-miRNA hairpins from the sequences adjacent to the mature miRNAs). This study provides a more detailed view of small RNA transcriptional landscape in MM, through a comprehensive analysis of RNA-seq data, coupled with microarray profiles, of a large panel of primary MM tumors.

Methods: RNA-seq data from 50 MM samples, representative of common cytogenetic alterations [hyperdiploidy (HD), 14q32 translocation, del(17), del(13), 1q+] were generated on Illumina HiSeq platform. After trimming and quality-based filtering, Bowtie was used to map reads and miR&more pipeline [Gaffo et al, Animal Genet 2014] applied to generate miRNAs and moRNAs expression matrices. DESeq and edgeR R/Bioconductor packages were used for differential expression analysis. MiRNA expression data of the same tumors were generated on Genechip¬Æ Human miRNA v3.0 array as previously described [Calura et al, Oncotarget 2016].

Results: Small RNA-seq of 50 MM samples led to 18.7 million average reads per sample. Of these, 9.7% on average were discarded after filtering. First, we demonstrated on coupled samples that RNA-seq and microarray data analyses led to >70% overlapping results in the detection of differentially expressed known miRNAs between subgroups. Marked upregulation of the miR-99b/let-7e/miR-125a-5p cluster was detected in t(4;14) patients by both approaches, whereas only RNA-seq unraveled the abnormal and specific expression of miR-135a-5p, most likely overcoming hybridization biases due to sequence similarity within miR-135 family. Overall, RNA-seq allowed characterizing 655 known miRNAs, 17 new miRNAs and 18 new moRNAs. While the 17 newly-identified miRNAs were weakly expressed, several moRNAs showed high and group-specific expression. Among these, the most significant were moR-150-3p and moR-21-5p and moR-941-5-5p in t(14;16)/t(14;20) samples, and moR,àí6724,àí1,àí5p in patients with HD.

Conclusions: RNA-seq of a representative panel of MM tumors confirmed and extended miRNA transcriptional profiling by microarray, outperforming this where sequence similarities might generate biases. Ultimately, we generated a more complete overview of small non-coding RNA in MM, unraveling also specific moRNAs that demand further investigations and candidating them, like miRNAs, as primary actors in MM biology.

C014

DIFFERENTIAL METHYLATION PROFILES IN PRIMARY PLASMA CELL LEUKEMIA PATIENTS STRATIFIED ACCORDING TO THE MAIN GENOMIC ABERRATIONS BY MEANS OF HIGH RESOLUTION MICROARRAY ANALYSIS

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Primary plasma cell leukemia (pPCL) is an aggressive and rare variant of multiple myeloma (MM) that is characterized by peculiar adverse clinical and biological features. Recently, significant changes in DNA methylation status were identified in association to MM disease progression and cytogenetic aberrations, prompting us to perform genome-wide methylation profiling in pPCL. Twenty-four pPCL cases at onset, almost all enrolled in GIMEMA clinical trial (lenalidomide-dexametasone combination), were previously analyzed for global gene expression profiling, by Affymetrix Gene 1.0 ST array. All pPCL cases were also characterized for the main chromosomal alterations by FISH analysis. Genome-wide methylation profiling data were generated in 14 pPCLs by Illumina Infinium 450 Beadchip array. Significant hypo and hypermethylated probes across the entire dataset (mean β-value <0.3 or >0.7) were evaluated in the context of their genomic localization. Differential methylated probes (dmps) were selected by means of Minfi Bioconductor package (q-value <0.05 and mean β-value difference >0.2). Pearson's correlation was applied between expression and methylation levels. A significant hypomethylation was observed in the 5'untranslated region (UTR5) and in the promoter gene regions, surrounding the transcription starting sites (TSS1500, TSS200, exon1), whereas higher methylation levels were evidenced in gene body and UTR3. Grouping of the 14 pPCL samples according to the most variable methylation probes seemed to be principally driven by the presence of the main IgH chromosomal translocations (trx), as t(11;14) or MAF-trx. Significant dmps were identified mainly in pPCLs patients with t(11;14), chromosome 1 aberrations (1p loss, 1q gain) or del(17). Particularly among them, PTGER4, a prostaglandin receptor whose stimulation is known to promote bone formation, resulted hypomethylated and highly expressed in t(11;14)-pos/1q gain-neg pPCL cases compared to pPCLs carrying poor prognostic genomic alterations (1q-gain, t(4;14) or MAF-trx). Furthemore, RNF165, found involved in proteasome protein catabolic process, resulted at higher methylation and lower expression levels in pPCLs with t(11;14). Significantly anti-correlated expression-methylation profiles were observed for both PTGER4 and RNF165 genes, across the entire pPCL dataset. Additionally, the NR2F2 gene, a nuclear receptor acting as a ligand

inducible transcription factor in the regulation of many different genes, showed higher methylation levels in pPCL cases with 1p-loss compared to 1p normal cases. Interestingly, NR2F2 was found aberrantly methylated in other hematological malignancies and particularly correlated with shorter survival in mantle cell lymphoma patients. Our data indicate that the integrative analysis of methylation-expression data may be helpful in the identification of novel potential molecular targets and can provide a contribution for the development of new therapeutic strategies in pPCL.

C015

INHIBITION OF ENDOTHELIAL AND PLASMA CELLS NOTCH MEDIATED COMMUNICATION REDUCES ANGIOGENESIS IN MULTIPLE MYELOMA PATIENTS

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Backgound: Multiple Myeloma (MM) is a hematopoietic malignant disease preceded by a Monoclonal Gammophaties of Undetermined Significance (MGUS) stage. Direct interaction of plasmacells (PCs) with endothelial cells (ECs) facilitates the tumor progression stimulating bone marrow angiogenesis. Mammals express four trans-membrane Notch receptors (Notch1-2-3-4), which bind five different ligands (DLL1-3-4 and Jagged1-2). Notch receptors regulate gene expression favoring proliferation, survival and embryonic development. γ-secretase receptor cleavage, mediated by cell interaction, is responsible of Notch pathway activation and, based on its involvement in angiogenesis, we first evaluate Notch basal expression level in ECs of MGUS and MM patients. Subsequently, we determine the role of Notch pathway in MMECs and tumor PCs direct crosstalk.

Materials and Methods: RT-PCR, western blotting and immunofluorescence were performed to assess both gene and protein Notch expression. Pathway modulation was evaluated in normal and co-culture conditions using siRNA designated for Notch1 and Notch2 receptors and γ-secretase (MK-0752) treatment through functional *in vitro* assays (adhesion, spontaneous migration, chemotaxis, capillarogenesis *in vitro* on Matrigel®).

Results: Notch1 and Notch2 mRNA expression levels were decreased of 30% and 50% in MMECs than MGECs respectively. Conversely, the relatively protein cleaved form was higher in MMECs than MGECs (50% for Notch1 and 70% for Notch2). Investigating on Notch activation, we found that its targeted genes Hey1 and Hes1 were highly expressed in MMECs compared to MGECs. Regarding functional assays, Notch1 and Notch2 inhibition reduced chemotaxis (40% and 35%), adhesion (10% and 50%), spontaneous migration and *in vitro* angiogenesis on Matrigel® in MMECs. In co-culture experiments, Hey1 expression was no significantly different between direct/indirect crosstalk. On the other hand, direct and indirect culture determined an increase of Hes1 expression both at 24h and 48h. Adhesion assay revealed that MK-0752 treatment decrease cells adherence capability of almost 20%. Moreover, chemotaxis reduction (20%), decreased spontaneous migration after wound, *in vitro* reduced angiogenesis on Matrigel® and inhibition of pro-angiogenic cytokines release were also noticed after MK-0752 treatment.

Conclusions: Due to the angiogenic modulation orchestrated by Notch we identify this pathway as an innovative target for the development of new drugs in MM therapy. Indeed, both the higher gene expression levels of Hey1 and Hes1 in MMECs and the higher activation of Notch1 and Notch2 receptors than MGECs confirm our hypothesis. In co-culture experiments we demonstrate an active role of PCs in enhancing ECs angiogenic abilities by stimulation of Notch pathway. On the other hand, siRNA silencing and treatments with γ-secretase negatively affect those acquired capabilities, suggesting the development of Notch inhibitors based therapies.

C016

MTOR INHIBITION AS ANTIANGIOGENIC STRATEGY IN MULTIPLE MYELOMA

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Backgound: The mammalian target of rapamycin (mTOR) is an intracellular ser/thr kinase that mediates intracellular metabolism, cell survival and actin rearrangement. mTOR is formed by two independent complexes i.e., mTORC1 and mTORC2 activated by the scaffold proteins RAPTOR and RICTOR respectively. It has been shown that cellular metabolism regulation is directly regulated by mTOR, confirming the hypothesis that its pathway dysfunction is associated with several type of cancer. Based on this scenario, we wanted to evaluate mTOR expression and activation in endothelial cells (ECs) of Monoclonal Gammophaties of Undetermined Significance (MGECs) and Multiple Myeloma (MMECs) patients and its possible involvement in MM angiogenesis.

Materials and Methods: Western blot and RT-PCR were performed to determine mTOR expression in MGECs and MMECs. To find out the role of mTOR in angiogenic progression *in vitro*, functional assays such as wound healing, chemotaxis, adhesion and Matrigel® assays were performed under RICTOR silencing and under treatment of both complexes inhibitor PP242. Angiogenic modulation was tested *in vivo* with CAM assay.

Results: MMECs showed a significant higher expression of mTOR (60%) and RICTOR (130%) than MGECs. RAPTOR expression, instead, was decreased by -90% in MMECs compared to MGECs. Those findings were also confirmed in immunofluorescence assay for RICTOR, RAPTOR and p-mTOR levels. In order to confirm mTORC2 activation, we screened for its downstream effector Akt. We found an increase of 50% in the Akt phosphorylation accompanied by a critical drop of phospo-S6K1 that indicates a block of mTORC1 pathway. Regarding functional assays, MMECs angiogenic capabilities were negatively regulated in a similar way in both siRNA and PP242 conditions. Indeed, chemotaxis and adhesion were reduced by -15%, chemoinvasion by -35%, with spontaneous migration and *in vitro* capillarogenesis that were strongly lowered. Moreover, PP242 showed a synergistic effect with lenalidomide and bortezomib, suggesting that mTOR inhibition can enhance the effect of those mentioned drugs. In conclusion, CAM assay revealed a significant reduction in new vessels formation number.

Conclusions: For the first time, we document that MMECs show a higher mTOR activation than MGECs. In this scenario, RICTOR and RAPTOR findings suggest a major role of mTORC2 in the "angiogenic switch". As both *in vitro* and *in vivo* results confirmed, mTOR inhibition highlights the mTORC2 pivotal role in MMECs angiogenesis. Introducing mTORC2 as a new target in MM treatment could enhance synergistic effects between different anti-MM drugs. Future experiments will be needed to define PP242 as a possible candidate for this purpose.

Chronic Lymphocytic Leukemia and Chronic Lymphoproliferative Disorders

C017

BCL2 AND BCL3 TRANSLOCATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA: PREVALENCE, ASSOCIATION WITH FISH AND PROGNOSTIC IMPACT

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Introduction: In chronic lymphocytic leukemia (CLL), recurrent chromosomal abnormalities, detected by fluorescent in situ hybridization (FISH), have been reported and stratified by the model proposed by Dohner et al. in 2000. However, FISH, focusing on specific genetic lesions, fails to detect additional chromosome abnormalities, which are detected by cytogenetic analysis only. Here we investigated the impact of BCL2 and BCL3 translocation in patients with CLL.

Methods: CLL cases with atypical morphology and low Matutes' score were excluded from this study. Cytogenetic analyses were obtained after 72 hours of stimulation with 500 μM CpG oligonucleotide and 20 U/mL of IL-2. Categorical variables were analyzed with Fisher exact test. Treatment free survival (TFS) was defined as time from diagnosis to first-line therapy (event) or last know follow-up (censored). Survival curves were estimated using the Kaplan-Meier method and differences in survival distribution were analyzed by Log-rank tests. All analyses were performed at a significance level of 5%.

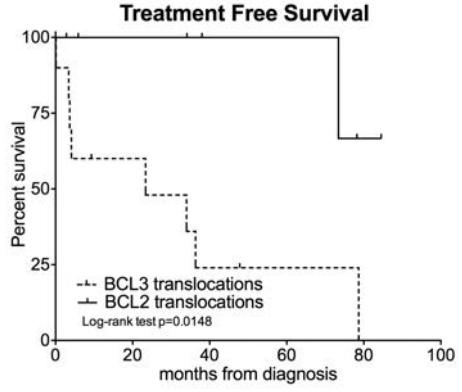

Figure 1.

Results: Ten patients (2.4%) out of 422 had recurrent translocations involving BCL3 and immunoglobulin (IG) locus, t(14;19)(q32;q13), of which 6 were male and the median age at diagnosis was 59 years. Instead, other 8 (1.9%) patients harbor translocations of BLC2 gene and heavy or light chain IG, t(14;18)(q32;q21) or t(2;18)(p12;q23) or t(18;22)(q21;q11). Among these patients 4 were male and the median age at diagnosis was 63 year. All these cases were characterized by typical morphologies and high Matutes' scores. Among patients with t(14;19), 2 harbor 13q deletion, 4 trisomy of chromosome 12 and 3 had normal FISH. Instead, among patients with BCL2 rearrangements, 6 were characterized by 13q deletion, 1 by trisomy of chromosome 12 and 2 had a normal cytogenetic by FISH. No patients would have been classified as high-risk CLL, given the absence of TP53 abnormalities.

After a median follow-up of 55 months, Kaplan-Meier analysis showed that median TFS for patients with t(14;19) was 23 months, while it was not reached for patients with BCL2 translocations. The estimated 5-year TFS were 24% and 100%, respectively (Log-rank test, p=0.0148, Figure 1).

Conclusions: We herein demonstrate that chromosomal translocations involving BCL2 and BCL3 are rare cytogenetic events in CLL and that t(14;19) was associated with an aggressive behavior characterized by a shorter time to first treatment, regardless to FISH hieratical model. This study emphasizes the usefulness of stimulated cytogenetic analyses to accurately define the prognosis of patients with CLL.

C018

PROGRESSIVE TELOMERE SHORTENING IS PART OF THE NATURAL HISTORY OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) AND IMPACTS CLINICAL OUTCOME: EVIDENCES FROM A STUDY ON PATIENTS WITH A LONG TERM FOLLOW-UP

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Introduction. Chronic Lymphocitic Leukemia (CLL) is one of the hematological disorders in which telomere biology has been extensively investigated. In CLL, telomere length (TL) < 5000 bp at diagnosis was associated to IGHV mutational status and to a poor outcome in terms of OS, TFS and progression to Richter's syndrome independently from the previously established predictors (cytogenetics, immunophenotype, IGHV-MS and p53 disruptions). However, data on TL dynamics over time are still scant and anecdotal. Aim of this study was to evaluate telomere kinetics in a retrospective series of CLL patients (pts)

Methods. Pts cohort was collected at University of Turin and University of Eastern Piedmont and characterized in terms of clinical and biological parameters. TL, assessed by Southern blot analysis, was analyzed at diagnosis and during the clinical CLL follow-up. Median telomere loss between the two determinations was calculated in terms of yearly loss (YL) and telomeric erosion based on baseline TL (%YL). Telomeric dynamics were compared to continuous variables by the Mann-Whitney test. Cut-off value for %YL was selected according to receiver operating characteristic (ROC) analysis, while only in the WW series, treatment-free survival (TFS) was calculated using the stratified Kaplan-Meyer method.

Results. CLL population(n=90), with a median clinical follow-up of 128 months (21-336 months), received a paired TL determination. The second TL determination was performed in 64 untreated pts, and in 26 pts at relapse. As expected, on the whole population telomeres were shorter at follow-up compared to baseline determination (median YL of 137 bp; range +174 bp; -1906 bp, p<0.001). Among any available clinical or biological predictors, a more pronounced erosion was observed in IGHV-mutated patients (YL -205bp vs 63 bp; p<0.05). YL was strictly dependent on baseline TL (YL -49 bp vs -129 bp for pts in the 25°and 100° TL percentile, respectively), so %YL was able to better describe telomeric dynamics in pts with short TL. Notably, in the 64 WW patients, a YL%

above 5.9% was predictive for an inferior median TFS (51 vs 163 months; p<0.007), despite being more common in pts with longer baseline telomeres and IGHV-mutated genes (theoretically a favorable group, figure1a). Also, we identified a super-favorable group of patients characterized by TL baseline >5000 bp and %YL under 5.9%, having a median survival of 179 months vs 53 months of other patients, having at least one telomere-related risk factor (p< 0.0005, figure1b).

Conclusions: our results on TL dynamics in CLL indicate the following: i) progressive telomere erosion occurs as part of the natural history of CLL; ii) telomere loss is more pronounced when baseline TL is higher; iii) accelerated telomeric loss associates to an inferior TFS. The results described in the present analysis corroborate basic studies suggesting that telomere disruption represents a critical step associated to CLL progression.

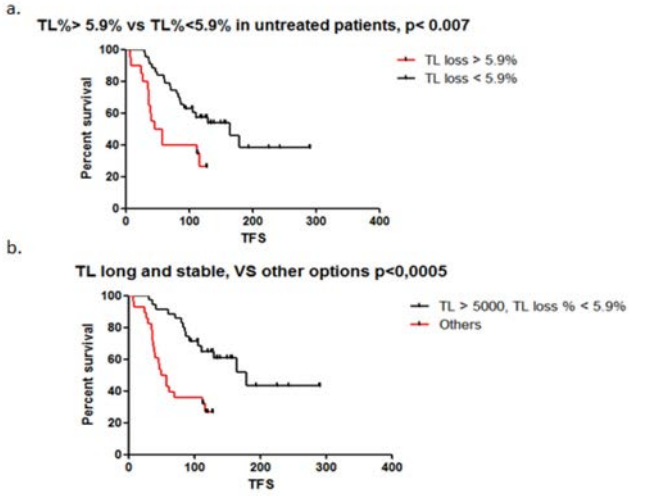

Figure 1.

C019

LNCRNA PROFILING IN EARLY STAGE CHRONIC LYMPHOCYTIC LEUKEMIA IDENTIFIES TRANSCRIPTIONAL FINGERPRINTS WITH RELEVANCE IN CLINICAL OUTCOME

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Despite its clinical indolent course, chronic lymphocytic leukemia (CLL) remains an incurable tumor. To establish the potential pathogenetic role of long non-coding RNAs (lncRNAs) in the disease, we investigated lncRNAs expression in a prospective series of 217 Binet stage A CLL patients and 26 different sub-population of normal B-cells, by using a custom annotation pipeline of Gene 1.0 ST microarray data. Specifically, we explored the expression of 1852 lncRNAs annotated in LNCipedia v3.1 that is currently the largest integrated repository of human annotated lncRNAs. To the best of our knowledge, this is the first report describing the global expression profiles of lncRNAs in a large cohort of samples representative of a homogenous series of early Binet A patients. Our study identified a 24-lncRNAs signature that is specifically deregulated in this pathology compared to normal B-cell counterpart. Importantly, this classifier was cross-validated on an independent dataset. In addition, we defined specific lncRNA signatures characterizing distinct molecular and genetic CLL groups. Some lncRNAs are recurrently associated with negative prognostic markers; in particular, lnc-AC004696.1-1 resulted up-regulated in poor prognostic group and negatively associated with PFS as well. LncRNAs interplay with genes and miRNAs in CLL was also investigated. To note, Lnc-AC004696.1-1 positively correlates with its antisense overlapping gene ZNF667. As regards miRNAs, we identified 11 lncRNA-miRNA couples with transcripts resulting anti-correlated and for which the lncRNA is a predicted miRNA target, based on one of the most common target prediction algorithms RNA-22. Finally, we evaluated whether a specific lncRNA signature was significantly associated with Progression Free Survival (PFS) in our prospective series. To this aim, a globaltest was run on the 471 lncRNAs whose expression varied mostly over the dataset. Among these, eight lncRNAs showed significant association with PFS (P<0.001).

In conclusion, our data extend the current view of lncRNA deregulation in cancer pointing to the potential relevance of the lncRNAs family in the context of CLL, which may contribute to discover novel putative molecular markers associated with the disease.

C020

BEPRIDIL IS A POTENT NOTCH1 INHIBITOR AND EFFICIENTLY INDUCES APOPTOSIS IN CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL) CELLS *in vitro* AND *in vivo*

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Introduction: NOTCH1 mutations represent one of the most frequent alterations in chronic lymphocytic leukemia (CLL),affecting up to 20% of patients. NOTCH1-mutated patients show poor outcome. In addition Notch activation modulates apoptosis resistance in this disease. Thus, the potential role of the NOTCH1 as a therapeutic target in CLL needs to be properly evaluated. Severe toxicity limited the clinical use of gamma secretase inhibitors (GSI) with anti-Notch activity in cancer. Recently, the calcium channel blocker bepridil demonstrated anti NOTCH1 modulating activity in T-ALL. Here we evaluated whether bepridil exerts antitumor activity associated with NOTCH1 inhibition in CLL cells.

Methods: We analyzed a gene expression data set of GSI treated CLL cells and compared it with different NOTCH-off signatures established in non-CLL malignancies. *in vitro*, we evaluated NOTCH1 expression in primary CLL cells after bepridil using western blot and flow cytometry. We measured apoptosis using annexin V/propidium iodide and assessing PARP, MCL-1 and NOXA expression. *In vivo*, CLL cells were transplanted into NSG mice and engraftment was evaluated after 28 days of bepridil treatment.

Results: We compared the NOTCH-off expression data of CLL with the transcriptional profiling of T-ALL after GSI inhibition. A gene set enrichment analysis revealed a significant enrichment across signatures with a subset of genes similarly regulated by Notch in CLL and T-ALL. This result was the rationale of repur-

posing in CLL the small molecule bepridil, identified in a gene-expression based small molecules screen for NOTCH1 inhibitors in T-ALL. Bepridil treatment significantly reduced CLL cells viability to 33.9±23.9% compared to 53.4.±23.9% of the vehicle (p<0.0001) without affecting B or T cells from healthy donors. Bepridil significantly increased annexin V/propidium iodide percentage compared to vehicle (39.9±21.9% vs 23.2±15.9 p<0.0001). Apoptotic effects depended on increased PARP degradation, reduction of MCL-1, NOXA up-regulation and were not correlated with NOTCH1 mutation. Flow cytometry demonstrated that bepridil reduced the surface expression of NOTCH1 in CLL cells (30.5±14.7% vs 51±14.7%, p=0.0007). Western blot showed down-regulation of the full-length NOTCH1 precursor, the trans-membrane and activated NOTCH1 while NOTCH2 protein level remained unchanged. Culture of primary CLL cells with stromal cells did not protect from bepridil-induced cytotoxicity. Finally, we established CLL NSG primagrafts and tested bepridil in this leukemia model. Strikingly, flow cytometry analysis revealed a significant decrease in human CD45+CD19+CD5+ cells in the spleen of bepridil-treated mice compared to vehicle (1.9±1% vs 10.8±10% p<0.05).

Conclusions: We showed antileukemic effects of bepridil in primary CLL cells that are associated with the inhibition of NOTCH1. *in vitro* and *in vivo* data suggest a potential for translation to clinical testing of these clinically relevant drug.

C021

THE BCR-DEPENDENT INSIDE-OUT ACTIVATION OF THE VLA-4 (CD49D/CD29) INTEGRIN IS MAINTAINED IN IBRUTINIB-TREATED CHRONIC LYMPHOCYTIC LEUKEMIA CELLS: IMPLICATION FOR RECIRCULATION LYMPHOCYTOSIS

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Background: Treatment with the B-cell receptor (BCR) signaling inhibitor ibrutinib (IB) has demonstrated high response rates and improved survival in chronic lymphocytic leukemia (CLL). It is associated with a highly variable degree of transient lymphocytosis, due to a reduction of CLL cell adhesion and efflux of CLL cells from tissue sites. CD49d, a strong negative prognosticator in CLL, is the alpha-chain of the integrin heterodimer CD49d/CD29 (VLA-4), a key molecule in CLL microenvironmental interactions. Inside-out activation of VLA-4 by BCR signals increases its adhesive properties, although regulation of VLA-4 activation by BCR triggering in CLL is unknown.

Aim. To correlate the kinetics of transient lymphocytosis with the expression and levels of activation of VLA-4.

Methods. The study included two cohorts of IB treated patients (IB-CLL), all with data of absolute lymphocyte count (ALC) at pre-treatment and at different time points of IB treatment: 1) Italian (IT) cohort (28 CLL patients, 15 CD49d+); 2) US cohort (26 CLL patients, 14 CD49d+). VLA-4 activation was assessed by flow cytometry using conformation sensitive anti-CD29 mAb HUTS-21 with LDV-containing VLA-4 specific ligand. The levels of activated VLA-4 was measured as VLA-4 receptor occupancy (RO) in values ranging from 0.0 (no RO) to 1.0 (100% RO) as in Chigaev et al. (J Biol Chem, 2009). Goat F(ab,Ä≤)2 anti-human IgM were used for BCR engagement. Ca++ release was cytometrically analysed. Adhesion assays were performed on VCAM-1-coated slides.

Results. Correlation between CD49d expression and IB-induced lymphocytosis was performed in both cohorts. Despite a different median ALC at baseline, CD49d- CLL showed a more pronounced increase in ALC than CD49d+ cases in both cohorts (Figure 1). To explain the observed differences in ALC kinetics in CD49d+ and CD49d- CLL, VLA-4 inside-out activation upon BCR stimulation was evaluated in CD49d+ CLL cells (n= 7) exposed in-vivo to IB. Despite an impairment of BCR-dependent Ca++ signaling (mean Ca++ release: 12.4% at pre-treatment, 3.6% at t30), IgM stimulation increased both VLA-4 RO (mean 0.53-range 0.40-0.73-, versus 0.36-range 0.22-0.52-, at pre-treatment, p=0.004; 0.53-range 0.41-0.84-, versus 0.27-range 0.01-0.45-, at t30, p=0.010) and CLL cell adhesion (mean values of adherent cells/control= 4.6 vs 3.7 at pre-treatment and 4.5 vs. 1.6 at day 30). Similarly, cells from IB-CLL on day 60-90 of treatment (n=3) showed an increased VLA-4 RO upon IgM stimulation (from 0.14, range 0.10-0.17 to 0.34, range 0.20-0.56), although Ca++ release was relevantly decreased.

Conclusions. BCR triggering in CLL cells activates VLA-4 via an inside-out pathway at least in part independent from IB binding to BTK. CD49d+ cells retain VLA-4 activation after IB treatment, with implications for CLL cell adhesion, and treatment-induced lymphocytosis. These observations should be considered in the design of IB therapies.

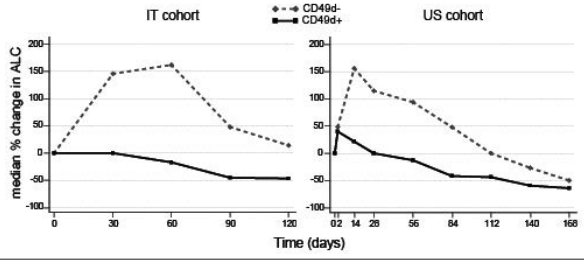

Figure 1.

C022

THE CONCOMITANT HIGH EXPRESSION OF THE B-CELL RECEPTOR SIGNALING INHIBITOR MOLECULES CD150, CD305, AND CD307B IDENTIFIES A VERY GOOD PROGNOSIS CHRONIC LYMPHOCYTIC LEUKEMIA SUBSET

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Introduction: Chronic lymphocytic leukemia (CLL) is characterized by clinical heterogeneity that can be observed also in the context of low-risk cases, thus the identification of novel markers that may predict an indolent clinical course can be of key clinical relevance. The CD150, CD305, and CD307b molecules have been independently reported as molecules associated with a mutated IGHV status, and with longer overall survival (OS) in CLL. However, the prognostic relevance of the combined CD150/CD305/CD307b expres-

sion in predicting OS in the context of low-risk CLL remains to be explored.

Methods: The study included 468 CLL cases characterized at diagnosis for Rai stage (stages 0-I: 358 cases), CD49d expression (CD49d- CLL, <30% of positive cells by flow cytometry: 257), IGHV mutational status (mutated, M: 271), karyotype abnormalities according to the hierarchical stratification (normal/13q-/+12: 366). Median follow-up of patients was 880 months with 82 deaths. Immunophenotypic analysis was performed in thawed samples on live cells.

Results: A significantly higher (p<0.0001) expression of CD150, CD305 and CD307b was documented in M versus UM CLL. The best cut-off levels for OS, calculated using a ROC analysis, were: 50% for CD150; 10% for CD305; 80% for CD307b. Using these cut-offs, 211 (45.1%), 332 (70.9%) and 331(70.7%) were classified positive for CD150, CD305 and CD307b respectively. The clinical impact of the three markers as OS predictors was confirmed in both univariate (p<0.0001 for CD150, CD305 and CD307b) and multivariate (hazard ratio/confidence interval HR/CI=0.36/0.20-0.66; p=0.0008 for CD150; HR/CI=0.50/0.32-0.77; p=0.0020 for CD305;HR/CI=0.40/0.25-0.63; p=0.0001 for CD307b) analyses. Therefore, we combined their expression in a 0 (all the three markers below the cut-off) to 3 (all the three markers above the cut-off) score, and dichotomized CLL cases according to the expression of 3/2 markers (n=307) versus 0/1 markers (n=161). The prognostic impact of this combined markers expression was tested in univariate analysis (p<0.0001) and in a multivariate model including: IGHV mutational status, CD49d expression, Rai stage (stage 0-I versus stages II-IV), karyotype abnormalities (normal/del13/+12 versus del11/del17). The combined markers expression retained its prognostic impact (HR/CI=0.44/0.26-0.75; p=0.003), along with the UM IGHV, CD49d+ expression, del11/del17. Moreover, expression of ≥2 markers was associated with a better prognosis in the context of the M IGHV (p=0.0009), CD49d- (p=0.0001), Rai 0-I stage (p<0.0001) and normal/del13/+12 karyotype (p=0.0001) groups.

Conclusions: High expression of at least two of the CD150, CD305 and CD307b molecules predicts longer OS in CLL, also in the context of low-risk prognostic categories. A synergic effect of the CD150, CD305 and CD307b molecules, all inhibitors of the B-cell receptor signaling, may be taken into account to functionally explain this peculiar clinical behavior.

C023

HIGH-THROUGHPUT SEQUENCING FOR THE IDENTIFICATION OF TP53 MUTATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. TP53 mutations are strong predictors of short survival and poor responses in chronic lymphocytic leukemia (CLL) and have important implications for patient management.

Methods. To analyze TP53 mutation in early stage Binet A untreated CLL (enrolled in the prospective O-CLL1 multicentre trial), we investigated by next generation sequencing (NGS) a cohort of 247 cases at onset, including 60 cases of clinical monoclonal B lymphocytosis (MBL), 130 of whom were also tested at 24/36 months or at progression. Moreover, we examined by conventional Sanger sequencing 13 additional clinical MBL patients and 27 CLL patients at onset. Deep sequencing of TP53 exons 4-9 on genomic DNA was performed by Roche 454 pyrosequencing on the Genome Sequencer Junior instrument and mutations were validated by conventional sequencing whenever the sensitivity of the Sanger method was consistent with the variant allele frequency (VAF).

Results. Globally, in the 287 tested patients we identified 15 non-synonymous somatic variants in 19 cases (6.6%). Variant allele frequency (VAF) obtained by deep sequencing ranged from 2.84% to 98.34% of total reads (median depth of coverage 225x, range: 48-1251). Among the 15 tumor-specific mutations, 14 (93%) were single nucleotide variations, while the remaining one (7%) was an indel. At the aminoacid level, 13 mutations (86%) were missense, one (7%) non-sense and one (7%) an in-frame deletion. All but one of these variants are listed in the dataset of somatic mutations in sporadic cancers of the IARC database. We evidenced that mutations cluster in the DNA binding domain; four mutations (R175H, I195T, P278R and R283C) were recurrent, each affecting two patients. In the two mutated samples longitudinally analyzed, TP53 mutation status was confirmed as found at diagnosis; notably, in one mutated case, carrying TP53 R175H mutation, the variant allele frequency increased from 16 to 73%. VAFs of around 100% were observed in a fraction of patients with 17q deletion, except for one case disomic for chromosome 17. In the remaining cases, i.e. the del(17p13) samples and those disomic for chromosome 17, the percentage of variant reads was suggestive of a mutation present in heterozygosis or in a small tumor subclone. Notably, all patients with 17p deletion (7/281; 2.5%) showed a concomitant mutated allele. In these cases, a biallelic inactivation/disruption of the TP53 gene can be postulated.

Conclusions. Our data provide evidence that mutations of TP53 are recurrent and more frequent than 17p deletion in early stage CLL and further support the notion that they may represent a marker of progression.

C024

AN *IN VITRO* MODEL OF HAIRY CELL LEUKEMIA TO CLARIFY ITS HISTOGENETIC DERIVATION FROM HEMATOPOIETIC STEM/PROGENITOR CELLS VERSUS MATURE B CELLS

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Introduction. We showed the BRAF-V600E mutation to be the key genetic lesion of HCL (New Engl J Med 2011;364:2305), a mature (post-)germinal center B cell tumor likely deriving from memory B cells. However, BRAF-V600E reportedly occurs in the hematopoietic stem/progenitor cell (HSPC) compartment of HCL patients (Sci Transl Med 2014;6:238). Yet, the fully blown HCL phenotype does not appear to develop until mutated HSCPs have traversed a long series of differentiation steps until the mature B cell stage. Whether complete development of the HCL identity requires additional genetic events along the way and/or a permissive epigenetic landscape specific of a particular cell differentiation stage, is not fully clear: on one hand inactivating mutations of CDKN1B and KLF2 are found in a minority (<20%) of HCL patients (Blood 2015;126:1005; Leukemia 2015;29:503), and on the other hand we showed that pharmacological blockade of BRAF-V600E in the established leukemic clone appears sufficient to erase most of the unique biological features of HCL (Blood 2015;125:1207).

Methods. To further elucidate this issue, we developed an *in vitro* model of HCL histogenesis through the expression of BRAF-V600E in normal human HPSCs and mature B cells. HSPCs, bulk

mature B cells and memory B cells were purified from healthy blood donors by MACS/FACS for CD34, CD19 and CD27, respectively, and were infected with viral plasmids containing only the GFP reporter transgene or also a BRAF-V600E transgene. Transduced (GFP+) cells were then FACS-sorted to high purity and monitored for: i) expression of BRAF and phosphoERK (a read-out of BRAF kinase activity) by Western blotting; ii) morphology changes by confocal microscopy after phalloidin staining of the F-actin-rich hairy projections; and iii) viability, by AnnexinV labeling of apoptotic cells.

Results: We overcame the technical challenge of introducing multiple transgenes of interest in hard-to-transfect/infect primary human mature B cells by optimizing a transduction method based on baboon-envelope pseudotyped lentiviruses (Blood 2014;124:1221). *in vitro* expression of BRAF-V600E consistently resulted in strong ERK phosphorylation and in considerable increase of cellular viability over time, both in HSPCs (n=4 donors) and mature blood B cells (bulk population: n=2; memory subset: n=3) (Figure). However, unlike HSPCs (n=2 donors evaluated), only bulk mature (n=2) and memory (n=3) B cells consistently developed the hairy morphology typical of HCL following expression of BRAF-V600E (Figure).

Conclusions: By establishing the first *in vitro* model of HCL histogenesis, our initial results support the view that: i) apoptosis inhibition by BRAF-V600E provides a fitness advantage to mutated HSPCs and their progeny down to mature B cells; ii) only at the latter stage the HCL phenotype driven by mutated BRAF is fully acquired, without needing additional genetic lesions but rather being licensed by an epigenetic state specific of mature/memory B cells.

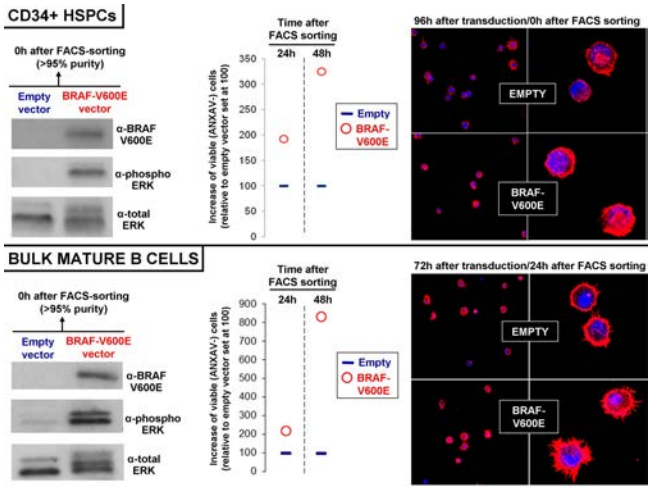

Figure.

Myeloproliferative Disorders and Chronic Myeloid Leukemia

C025

TRANSCRIPTIONAL PROFILE OF CD34+ PROGENITOR CELLS ACCORDING TO JAK2 OR CALR MUTATIONS IN ESSENTIAL THROMBOCYTHEMIA

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Introduction: Philadelphia-negative myeloproliferative neoplasms (MPNs) are a heterogeneous group of clonal stem cell disorders with common molecular and clinical characteristics, and include polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). 95% of patients with PV and 60% with PMF and ET harbor the somatic mutation JAK2V617F. In addition, mutations of MPL in exon 10 are present in about 5% of cases with ET or PMF. In 2013, somatic mutations in Calreticulin (CALR) gene have been reported in 50-70% of JAK2 and MPL-negative MPNs. The clinical course of CALR-mutated patients appears to be more indolent than that of JAK2-mutated patients. Moreover, they show relevant differences in terms of clinical parameters (thrombotic risk, platelet count, white blood cell count, haemoglobin level, etc.) compared with JAK2V617F patients (Rumi et al, Blood 2014).

Methods: We performed gene and miRNA expression profiling (GEP and miEP, respectively) in bone marrow-derived CD34+ cells from 26 PV, 29 ET patients and from 15 healthy donors (CTRs) by means of Affymetrix technology (HG-U219 Array Strip and miRNA 2.0 arrays). Among the 29 ET patients, 7 (24.1%) were CALR-mutated, 17 (58.6%) were JAK2V617F-positive, and 5 (17.3%) were triple negative; all PV patients were JAK2V617F-positive.

Results: The principal component analysis (PCA) performed on GEP and miEP data showed that PV and ET samples clustered together and were clearly separated from CTRs. Consistently with PCA results, gene set enrichment analysis (GSEA) demonstrated that the majority of modulated transcripts in PV or ET samples versus CTRs are shared between the two diseases. However, PCA performed on GEP and miEP data showed that CALR-mutated ET samples were clearly separated from both JAK2V617F-positive ET and PV groups, which instead clustered together. Thus, the comparison of GEP/miEP from CALR-mutated versus JAK2V617F-positive ET patients showed 2066 differentially expressed probesets distinguishing ET patients on their mutation status; conversely we didn't find any modulated gene in the pairwise comparison between PV and JAK2V617F-positive ET samples. The functional analysis of modulated transcripts unveils several pathways differentially activated between JAK2V617F- and CALR-mutated progenitor cells, i.e. mTOR and CSNK1A1 pathways. Moreover, in CALR-mutated progenitors we found the down-modulation of several genes involved in thrombin signalling and platelet activation that could explain the low risk of thrombosis in CALR-mutated patients in spite of their high platelet count.

Conclusions: This study supports the pathogenetic model in which JAK2V617F-positive ET and PV are considered as different phe-

notypes/phases of a same MPN, whereas CALR-mutated ET seems to be a distinct clinical and molecular entity (Cazzola *et al*, Blood 2014).

C026

NEXT GENERATION SEQUENCING (NGS) VERSUS SANGER SEQUENCING (SS) FOR BCR-ABL KINASE DOMAIN (KD) MUTATION SCREENING: FIRST RESULTS OF THE "NEXT-IN-CML, ITALIAN MULTICENTER STUDY

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Introduction: Some retrospective studies have shown that in Ph+ leukemia pts BCR-ABL mutation status may be more complex than SS shows, and that a proportion of pts with 1st-line treatment failure may harbor low burden TKI-resistant mutations detectable by NGS only. The frequency and clinical relevance of low burden mutations remains to be explored prospectively in larger series of pts. We have initiated a multicenter, multilaboratory prospective study ('NEXT-IN-CML') aimed to assess the feasibility, performance and informativity of a NGS-based BCR-ABL KD mutation screening approach.

Aims. The 1st phase of the study was aimed to set up and standardize a network of 5 Italian labs performing NGS of the BCR-ABL KD. The 2nd phase of the study, involving 54 Italian Hematology Units referring to one of the 5 labs, is aimed to assess the frequency and clinical significance of low burden mutations detectable by NGS in samples of CML pts with failure or warning and of relapsed/refractory Ph+ ALL pts.

Methods: A PCR and an amplicon NGS protocol already set up and optimized for the Roche GS Junior in the framework of the IRON II international consortium was adopted. In the 1st phase, 5 batches of 16 blinded cDNA samples were prepared and shipped to evaluate individual lab performances. The batches included 11 pt samples with known BCR-ABL mutation status and 5 serial dilutions of BaF3 T315I-positive cells in BaF3 unmutated cells, simulating mutation loads of 20% down to 5%. In the ongoing 2nd phase, samples are being analyzed in parallel by SS and NGS. Clinical history and follow-up data are being collected for correlations.

Results: 311/320 amplicons were successfully generated and sequenced. A median of 124,686 high quality reads – far exceeding the throughput of the instrument (80,000) – were generated by the 5 labs (range, 48,181-170,687). Sequencing depth was pretty uniform across amplicons and indexes. Median depth per amplicon was 3,568 reads (range, 773-14,334). Comparison of observed vs expected mutations showed that 76/78 samples evaluable by NGS were accurately scored (the 35INS was missed in 2 samples). Quantitation of mutation burden was highly reproducible for point mutations, less for the 35INS. The T315I dilutions simulating the lower mutation loads were quantitated accurately and reproducibly across all labs. So far, 61 samples from 52 CML and 9 Ph+ ALL pts have been included in the study. Low burden TKI-resistant mutations have been found in 6 pts. Updated results will be presented.

Conclusions: - NGS protocols for BCR-ABL KD mutation screening can successfully be shared and applied within networks of labs – including labs approaching the NGS technology for the first time; - high concordance in point mutation detection by NGS vs SS and between labs can be achieved; - NGS may find TKI-resistant low burden mutations in some samples. Follow-up data of the pts enrolled in the study will cast light on the clinical relevance of such mutations.

C027

MUTATIONS IN JAK2 AND CALRETICULIN GENES ARE ASSOCIATED WITH SPECIFIC ALTERATIONS OF THE IMMUNE SYSTEM IN MYELOFIBROSIS

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Introduction: Myelofibrosis (MF) is a clonal neoplasia associated with chronic inflammation due to aberrant cytokine production. MF patients have severely reduced life expectancy, with infectious complications constituting more than 10% of all causes of death. Although mutations in Janus Kinase 2 (JAK2), calreticulin (CALR) and myeloproliferative leukemia protein genes have been discovered, they all highly activate the JAK/STAT signaling pathway. Since JAK/STAT pathway is essential in shaping the immune response, we evaluated key immune-cell subsets and plasma cytokine levels in MF according to mutation status. In particular, we evaluated circulating Dendritic cells (DCs) and monocytes, focusing on their DCs differentiation capacity. DCs promote T cell polarization into different subsets of T-helper cells (Th) responsible for the clearance of intra and extracellular pathogens. For these reasons, we analysed the percentages of Th1, Th2 and Th17 cells; in addition, the role of natural regulatory T cells (Tregs) was investigated. Due to the aberrant cytokine compartment in MF, we also evaluated Innate Lymphoid Cells (ILCs), a novel family of immune effector cells with a critical role in inflammation and immunosurveillance.

Methods: Mutations were monitored using RT-PCR (JAK2 gene) and exon 9 Next Generation Sequencing approach (CALR gene). We evaluated circulating Th1, Th2 and Th17 cells, myeloid and plasmacytoid DCs from 30 untreated patients (20 JAK2(V617F) and 10 CALR mutated patients) and 20 healthy subjects by flow cytometry. Percentages and function of total Tregs and ILCs plus their cognate subpopulations were analysed as well. After immunomagnetic isolation, we tested phenotype of circulating monocytes and their capacity to differentiate into DCs. Cytokine plasma levels were measured by ELISA.

Results: We found that MF patients were characterized by reduced capacity of monocyte differentiation into DCs, decreased Th17 plasticity and hypo-functional ILCs. Furthermore, all patients showed a reduced plasma level of interleukin (IL) -4, -5 and Interferon-γ with concomitant increase of IL-1β, -6, -12, -13, -17, and Tumor Necrosis Factor-α. Despite these common defects, we identified a mutation-driven immunological signature. Th17, myeloid-DCs and effector Tregs reduction, plus an increase in ILC1, were specific of JAK2(V617F) mutated patients. Alternatively, CALR mutated patients presented increased ILC3 population, diminished Th1 compartment and a reduced monocyte-derived DC capacity to mature *in vitro* into a committed DCs. Moreover, Tregs from

CALR mutated patients were less suppressive than the normal counterpart, due to the increased *in vitro* proliferative capacity of responder T cells carrying CALR mutation.

Conclusions: MF patients are characterized by a mutation-driven state of immune alteration which may play a role in disease pathogenesis and infection susceptibility.

C028

ROLE OF C-MAF-INDUCED INFLAMMATORY MEDIATORS IN PRIMARY MYELOFIBROSIS PATHOGENESIS

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Introduction: Primary Myelofibrosis (PMF) belongs to BCR-ABL negative Myeloproliferative Neoplasms (MPNs), a set of clonal stem cell-derived hematological malignancies. PMF is characterized by a progressive expansion of the megakaryocytic lineage, development of myelofibrosis, osteosclerosis and mobilization of CD34+ hematopoietic progenitor cells (HPC). The disruption of the bone marrow (BM) structure is partially linked to a deregulated cytokines production. In order to better characterize the molecular mechanisms underlying PMF pathogenesis, we recently performed the Gene Expression Profiling (GEP) of HPC from PMF patients and healthy donors. The transcription factor c-MAF resulted upregulated in PMF samples and was selected for further experiments since it is overexpressed in Multiple Myeloma.

Methods: In order to study the role of c-MAF in hematopoietic differentiation, we overexpressed c-MAF in cord blood (CB) CD34+ cells by means of retroviral transduction to reproduce the condition observed in PMF HPC. We evaluated c-MAF effects by immunophenotypic and morphological analysis. To further characterize c-MAF overexpression effects, we performed the GEP of c-MAF-overexpressing CD34+ cells compared to control samples transduced with the empty vector LXIDN. With the aim to identify novel PMF biomarkers and to correlate them with c-MAF overexpression, we selected a set of secreted molecules upregulated in c-MAF-overexpressing cells and assayed them by quantitative enzyme-linked immunoassay (ELISA) in plasma samples from PMF patients (n=30) and healthy donors (n=10), together with cell culture supernatant samples from c-MAF-overexpressing and empty vector-transduced CD34+ cells (n=6). Finally, we evaluated the fibrogenic effect of these secreted molecules by the treatment of normal human dermal fibroblasts (NHDF).

Results: Our data demonstrated that c-MAF overexpression in CB CD34+ cells promotes megakaryocyte and monomacrophage differentiation, according to what observed in PMF patients. c-MAF overexpression causes the upregulation of several secreted molecules like interleukin 8, chemokine CCL2, metalloproteinase MMP9 and uPAR receptor, that are all at higher levels in PMF plasma compared to healthy donors. Lectin LGALS3 and osteopontin (OPN), which have never been correlated before to PMF, were increased both in PMF plasma and in c-MAF-overexpressing cell supernatants. In particular, increased OPN plasma levels in PMF patients correlates with a more severe fibrosis degree and inferior overall survival. We also observed that OPN promotes fibroblasts proliferation *in vitro*.

Conclusions: Our data supported the contribution of c-MAF in BM stromal changes typical of PMF, such as fibrosis, through the induction of inflammatory mediators. OPN could be considered a novel putative plasma marker in PMF patients. Further studies need to be performed to identify the cellular types responsible for the overproduction of inflammatory molecules.

C029

IN VITRO CHARACTERIZATION OF MESENCHYMAL STROMAL CELLS (MSC) FROM PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS (PH-NEG MPN): A COMPARISON BETWEEN HIGH AND LOW BONE MARROW FIBROUS GRADE GROUPS

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MSC play an important role in the structure and regulation of the bone marrow (BM) niche. In Ph-neg MPN there is a substantial alteration of the BM niche, characterized by deposition of fibrous tissue, neoangiogenesis, osteosclerosis and loss of the hematopoietic tissue. MSC were characterized after isolation from BM biopsies of 23 patients with Ph-neg MPN and BM harvests of 6 healthy donors (HD), from washouts of transplant bags. In order to collect mononuclear cells (MNC), BM fragments were digested using collagenase solution. Patients were classified in 3 groups according to diagnosis of Ph-neg MPN and grade of BM fibrosis (WHO criteria): low fibrosis (0-1) no MF (LF-NOMF), low fibrosis MF (LF-MF) and high fibrosis (2-3) MF (HF-MF), respectively formed by 11, 4, and 8 patients. Mutations of JAK2, CALR and MPL genes were present respectively in 65%, 22% and 4% of cases. A triple negativity was documented in two cases (9%).

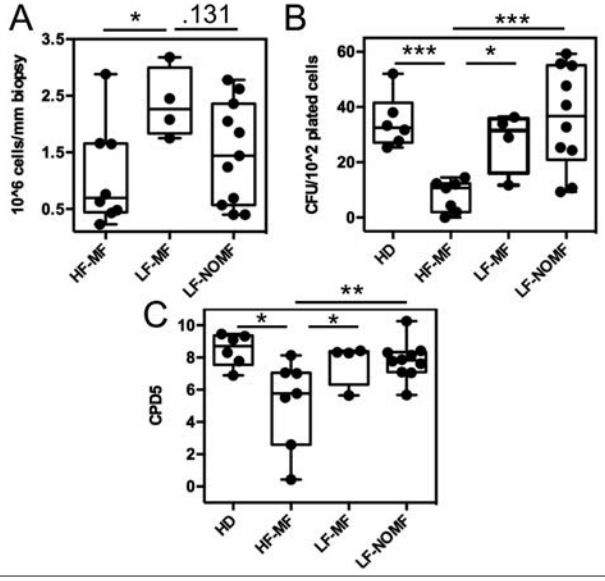

Figure 1.

The median age at the time of cell collection was 54 years (range: 38-77) for the patients and 50.5 years (44-58) for the HD group. A different number of MNC was harvested from biopsies digestion in different patients groups with a median value of 0.70x10^{6}cells/mm (0.23-2.88) for HF-MF and 2.27x10^{6}cells/mm (1.75-3.18) for LF-MF

(p 0.028). LF-NOMF cellularity was 1.44x10^{6}cells/mm (0.40-2.78). No difference was noted between the two groups with LF (p 0.131) (Figure 1A). Median value of CFU per 10^2 plated cells was 10.70 (0-14.50) for HF-MF, significantly reduced compared to the other groups: LF-MF 31.50 (11.70-36.30; p 0.042), LF-NOMF 36.70 (9.30-59.70; p 0.0049) and HD 32.50 (25.30-38.00; p 0,0012) (Figure 1B). The median cumulative population doubling (PD) value at passage 5 for HF-MF was 5.78 (0.43-8.14), significantly reduced compared to other groups: LF-MF 8.31 (5.66-8.42; p 0.05), LF-NOMF 7.83 (5.68-10.26; p 0.0097) and HD 8.71 (6.90-9.46; p 0.014) (Figure 1C). All patient's MSC lines express the typical markers of mesenchymal cells (CD90, CD105, CD73, CD146), similarly to HD. Regarding differentiation potential, Ph-neg MPN-MSC showed a normal differentiation capacity into adipogenic and osteogenic tissue, with an up-regulation of key differentiation genes (FABP4, LPL, PPARG for adipogenic lineage and RUNX2, ALPL, COL1A2, SPP1, SPARC, BGLAP for the osteogenic one) with a similar trend compared to HD. Ph-neg MPN-MSC were also able to differentiate into cartilagineous pellets, showing an up-regulation of key differentiation genes (COL2A1, COL10A1, SOX9, ACAN) similar to HD. Finally, MSCs were tested for the presence of the respective HSC mutations (JAK2, MPL, CALR) and no one harbored them.

Ph-neg MPN-MSC with HF showed a reduced clonogenicity and proliferation capacity compared to the LF groups, independently from the type of disease. These data suggest an alteration of MSC of Ph-neg MPN restricted to the MSC derived from patients who already have a HF in the BM.

C030

CAL2 MONOCLONAL ANTIBODY IS A RAPID AND SENSITIVE ASSAY FOR THE DETECTION OF CALRETICULIN MUTATIONS IN ESSENTIAL THROMBOCYTHEMIA AND MAY PROVIDE PROGNOSTIC INFORMATIONS

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Background. Calreticulin (CALR) mutations are detected in the majority of JAK2 wild-type (wt) patients with Essential Thrombocythemia (ET) or Primary Myelofibrosis (MF). At variance with JAK2 and MPL point mutations, CALR mutations are highly heterogeneous, with several types of insdel reported to date, so that sequencing techniques are needed to cover the whole spectrum of mutations. CAL2 is a new monoclonal antibody which specifically recognizes the C-neoterminal peptide derived from all the frameshift mutations of CALR.

Methods. We retrospectively analyzed 172 ET patients followed at our Institution from 1980 to 2015 who were tested for JAK2V617F at the time of diagnosis or during follow-up. In JAK2wt patients exon 9 CALR mutations were searched by PCR and capillary electrophoresis and MPLW515L/K by ARMS-PCR. In the same patients, bone marrow (BM) biopsies were immunostained with CAL2 and histologically reviewed for megakaryocytic features.

Results. Median age at diagnosis was 54 years (range 14-87) and median time from first evidence of thrombocytosis to BM biopsy was 8.4 months (range 0-175). According to driver mutations, patients were classified as JAK2V617F-mutated (n=119, 69%), MPL-mutated (n=2, 1%), CALR-mutated (n=31, 18%) or triple-negative (n=20, 12%). As expected, CALR-mutated patients had significantly higher platelet count than JAK2V617F (799 vs 657 x 10^9/L, respectively; p=.019) and lower hemoglobin values (mean 13.6 vs 14.5 g/dL, respectively; p=.003). At a median follow-up of 9.8 years, incidence of thrombosis was significantly lower in CALR- than in JAK2-mutated patients (3% vs 21%, respectively; p=.017), while no differences were observed in clinical presentation and long-term outcome between CALR-mutated and triple-negative patients. Concordance between molecular and immunohistochemical (IHC) detection of CALR mutations was optimal (Cohen's kappa >0.8) but not complete, since 3 patients were positive by IHC only and 1 patient was positive by molecular only. In CAL2-positive BM samples (n=30) we defined 2 patterns (figure), characterized by staining of megakaryocytes only (pattern A, 37%) or staining of megakaryocytes and myeloid precursors (pattern B, 63%). Type B biopsies tended to have higher median cellularity (48% vs 30%; p=.22), higher median megakaryocytic number for HMF (20 vs 12.5; p=.14), higher frequency of megakaryocytic clusters (100% vs 60%; p=.014) and higher frequency of grade-1 fibrosis (81% vs 60%; p=.37) with respect to type A samples. One type A patient and 2 type B patients progressed to post-ET MF or acute leukemia. Moreover, 3 patients had a BM biopsy performed at the time of MF evolution but not at diagnosis of ET, and they displayed a type B pattern.

Conclusions. CAL2 identifies CALR-mutated ET patients in a rapid, sensitive, specific and economic manner and it integrates molecular biology informations. Prognostic value of different IHC patterns should be confirmed in wider and indipendent series.

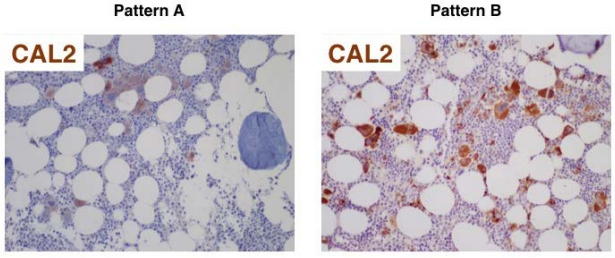

Figure 1.

C031

REGULATIVE LOOP BETWEEN β-CATENIN AND PROTEIN TYROSINE PHOSPHATASE RECEPTOR TYPE γ (PTPRG) IN CHRONIC MYELOID LEUKEMIA

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Introduction. Chronic Myeloid Leukemia (CML) is a myeloproliferative disease characterized by the presence of the oncogene BCR-ABL1, which acts as tyrosine kinase. PTPRG (Protein Tyrosine Phoshatase Receptor type γ) is a tumor suppressor gene down-regulated by hypermethylation of its promoter region in CML. Previous studies demonstrated that a re-expression of PTPRG is correlated with a decreased clonogenic capability of CML cells, as shown by the down-regulation of Ki67, and with an increased cellular differentiation related to a PTPRG-mediated overexpression of GATA-1 and Cyclin D1. In addition, its restored expression was observed in patients with a good response to TKI therapy. In order to understand the regulation between this phosphatase and BCR-ABL1, we searched for PTPRG putative interactors among proteins downstream BCR-ABL1 driven pathways and we focused on β-Catenin, that is at the same time a PTPRG substrate and its transcriptional regulator.

Methods. Cells: PTPRG negative cell line K562, with a stable transfection of exogenous PTPRG, and PTPRG positive cell line LAMA-84, treated with a specific siRNA and with a new PTPRG small drug inhibitor. Pull-down assay with purified, recombinant

intracellular domain of PTPRG demonstrated a direct interaction between PTPRG and β-Catenin, while Western Blotting or Immunofluorescence were applied to detect a specific dephosphorylation pattern in presence of PTPRG. Chromatin Immunoprecipitation showed us the binding between DNMT1(β-Catenin transcriptional target) and PTPRG promoter region.

Results. We demonstrated that PTPRG binds and dephosphorylates β-Catenin, phosphorylated by BCR-ABL1, causing its cytoplasmic destabilization and the resulting degradation in CML cell lines with an exogenous or endogenous expression of PTPRG (K562 and LAMA-84 cell lines). Consequently, this regulation leads to MYC down-expression and p21/WAF1 increased expression, explaining the slow-down of proliferation in presence of PTPRG. On the contrary, we demonstrated that an increased expression of β-Catenin in PTPRG negative CML cell lines is correlated with an over-expression of the DNA (cytosine-5)-methyltransferase 1 (DNMT1) that is responsible of PTPRG promoter hypermethylation and that an inhibition after a treatment with 5-Azacydine or a down-regulation of this enzyme is closely related to PTPRG re-expression both at mRNA and protein levels.

Conclusions. We show for the first time a mechanism that involves β-Catenin degradation control and the consequent down-regulation of genes regulated by the TCF/β-Catenin transcription complex. In return, β-Catenin up-regulation is correlated with an over-expression of DNMT1 that contributes to an hypermethylation of PTPRG promoter region. We hypothesized a regulative loop between PTPRG and β-Catenin and that an imbalance of the system in favor of one or the other could determine a different proliferation fate of CML cells and their clinical aggressiveness.

C032

STUDY AND MOLECULAR CHARACTERIZATION OF PATIENTS WITH SYSTEMIC MASTOCYTOSIS

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Introduction: Systemic Mastocytosis (SM) identifies a group of rare and clinically heterogenous myeloproliferative neoplasms characterized by abnormal growth and activation of mast cell and their precursors in the bone marrow and in various tissues. Disease variants can be recognized ranging from indolent SM (ISM) to aggressive SM (ASM) and SM with associated clonal hematological non-mast cell disease (SM AHNMD). KIT D816V is the driver mutation in SM and it is usually detected in bone marrow (BM) mast cells of SM patients (pts). Recent studies reported that, using an high sensitivity assay, the D816V mutation can be detected even in leukocytes from peripheral blood (PB). The presence of multiple additional mutations in other myeloid relevant genes was recently reported in > 90% of pts with KIT D816V+ SM (Schwaab *et al.* Blood 2013). We analyzed pts with mastocytosis with regard to the presence of mutations in genes commonly mutated in myeloid neoplasms, in addition to KIT, and their prognostic impact.

Methods: We analyzed 58 pts (46 ISM, 5 ASM, 6 SSM, 1 SM AHNMD), with an already known mutational profile of D816V by Real Time PCR, on cDNA (Lawley *et al.* 2005) and genomic DNA (Kristensen *et al*. 2011). We investigated the same cohort by next generation sequencing (NGS) using the Ion Torrent PGM platform with a gene panel that includes the most commonly involved genes in mastocytosis: c-KIT, ETNK1, TET2, CBL, IDH1, IDH2, SRSF2, ASXL1 and RUNX1. Statistical analyses were conducted with SPSS software.

Results: The Real Time PCR assay on cDNA showed that 54/58 pts (93%) resulted KIT mutated in bone marrow compared to 30/58 (51.7%) by Sanger sequencing: 23/54 (42.5%) BM mutated pts were also positive on cDNA PB. Pts negative for D816V in PB samples were further analyzed using genomic DNA: 27/31 (87%) resulted positive in the PB sample, unlike 4 (13%) who were positive only in the BM sample. Comparing NGS and Real Time PCR on genomic DNA, the second appears to be more sensitive: 9/58 were D816V positive using a filter at 5% on the allelic burden, while by setting the filter at 0.03% (the sensitivity achieved by the Real Time PCR) 41/58 resulted D816V positive. Above all, 10 pts showed several mutated genes, in addition to D816V: mutations mainly affected TET2 (10,3%), ASXL1 (1.8%), CBL (1,8%), SRFSF2 (1,8%), IDH2 (1.8%) genes. These mutations appeared to be associated with ASM (75%). Statistical analyses showed that mutations in addition to D816V were predictors of disease progression to a more aggressive form (p=0.0001) and to have a negative impact on prognosis (p=0.0001) because 75% of pts who died at last follow up were those having more than one mutation.

Conclusions: The most useful and feasible method for the diagnostic laboratory routine is the real-time PCR on genomic DNA that has a 0.003% sensitivity. In addition, analysis of an extended panel of myeloid neoplasm-associated mutations may provide prognostically relevant information.

Lymphomas

C033

HLA-G EXPRESSION IN ADVANCED-STAGE CLASSICAL HODGKIN LYMPHOMA

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Non-classical human leucocyte antigen (HLA)-G class I molecules have an important role in tumor immune escape mechanisms. We investigated the expression of HLA-G in lymphonode biopsies from patients with advanced-stage classic Hodgkin Lymphoma (cHL) in relationship with the achievement of negative results for positron emission tomography carried out after 2 cycles of standard chemotherapy (PET-2), and with HLA-G allelic variants characterized by a 14-basepair (14-bp) deletion-insertion polymorphism located in exon 8 of the 3'-untranslated region of HLA-G. Tissue lymphonodes sections obtained at the diagnosis from 20 patients with cHL and from 8 controls were stained using a specific murine monoclonal HLA-G antibody. HLA-G protein expression was higher in lymphonodes tissues from cHL patients than controls (Figure 1). In the group of PET-2 positive patients and a lower 2-years progression free survival (PFS = 40%), we observed a higher HLA-G protein expression on tumor microenvironment and a lower expression on HRS cells. Conversely, PET-2 negative patients with a better 2 years PFS (86.5%) had higher HLA-G protein expression levels on HRS cells compared to the microenvironment. Finally we found a significant association between the HLA-G insertion/insertion 14 –bp genotype and lower expression of HLA-G on HRS cells. Higher HLA-G expression in the tumor microenvironment surrounding HRS cells could favor an immune-escape mechanism, whereas higher HLA-G expression in HRS cells could offer better control of tumor proliferative activity. Hence, HLA-G could exert a dual role: i) tumor escape mediated by HLA-G-driven inhibitory mechanisms of antitumor response; and ii) tumor antiproliferative function. According with this hypothesis, the final effect on tumor progression might derive from the balance between these two mechanisms. New insights on HLA-G expression may enforce the rationale for developing innovative and targeted treatments in cHL based on the modulation of HLA-G function.

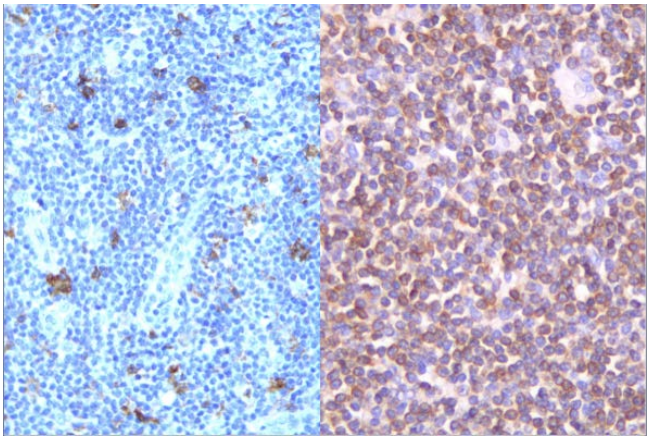

Figure 1. Immunohistochemical staining of HLA-G expression (brown color) in a control (left) and in an advanced-stage cHL patient (right) with a larger number of immunopositive reactive cells and a Reed-Sternberg cell.

C034

THE PI3K δ/γ INHIBITOR RP6530 SUPPRESSES IN VIVO HODGKIN LYMPHOMA GROWTH BY INHIBITING NEOANGIOGENESIS AND TUMOR ASSOCIATED MACROPHAGES

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Introduction: The phosphatidylinositol 3-kinase (PI3K) pathway plays a critical role in mediating tumor and endothelial cell survival and function. As PI3Kδ and PI3Kγ are active in many hematologic malignancies, resulting in cancer development and progression, we suggest that the novel dual PI3K δ/γ inhibitor RP6530 might represent a unique therapeutic opportunity for Hodgkin lymphoma (HL) patients. This study aimed to investigate the antitumor effect of RP6530 in preclinical HL models.

Methods: To identify the effect of RP6530 on HL cells (L-540, KM-H2 and L-428), cell proliferation and cell death assays, gene expression profiling, and Western blotting were conducted. The antitumor efficacy and mechanism of action of RP6530 was finally analyzed in NOD/SCID mice bearing HL cell line xenografts. Tumor vasculature was analyzed by *in vivo* biotinylation of vascular endothelial proteins.

Results: Exposure of HL cell lines to RP6530 (1.25 - 10 μM) induced a marked, early and dose-dependent dephosphorylation of PI3K/Akt and MAPK pathways that was associated with cell proliferation inhibition (L-540: 40 ± 4%, KM-H2: 33 ± 5% and L-428 30 ± 5%, mean ± SEM) and S phase cell cycle arrest (3-fold reduction, compared to vehicle-controls). Significant levels of caspase-dependent cell death were observed in L-540 (20 μM: 61 ~ 5 vs. 15 ~ 2%), KM-H2 (20 μM: 41 ~ 4 vs. 12 ~ 1%) and L-428 (20 μM: 31 ~ 4% vs. 12 ~ 1%) cells, associated with mitochondrial dysfunction (up to 40%). Interestingly, microarray gene expression analysis of KM-H2 cell line indicated that RP6530 treatment triggered, as early as 24 hours, decreased amplitude in "PI3K signaling in B lymphocytes", "Tumor cell proliferation", "Tumor angiogenesis", and "Macrophages activation" signatures, as well as induced upregulation of genes involved in positive regulation of cell death. Consistent with the role of PI3K signaling in the macrophage-dependent promotion of tumor growth and tumor-induced angiogenesis, we detected a severe reduction of tumor-associated macrophages (-16% and -27%, P ≤.0001 in L-540 and KM-H2, respectively, compared to vehicle-controls), and tumor vessel density (average 80% decrease) in RP6530-treated tumor xenografts. This finding was paralleled by histological observation of tumor vasculature disruption, and inhibition of phospho-Akt and -Erk on both endothelial and tumor cells. Furthermore, RP6530 (150 mg/kg/BID/3 weeks) treatment significantly reduced the growth of KM-H2 nodules (-60%, P ≤.0001 compared to vehicle-controls), associated with a strong decrease in Ki-67 expression in tumor cells, increase in tumor apotosis (1.2-, 12-fold in L-540 and KM-H2, respectively) and necrosis (mean 7-fold increase, P ≤.0001).

Conclusions: RP6530 inhibits PI3K/Akt and MAPK pathways via apoptosis, anti-angiogenesis and anti-macrophage recruitment. Based on these preclinical data, a Phase I, first-in-human, study evaluating the clinical activity of RP6530 in HL is currently ongoing.

C035

THE PROGNOSTIC VALUE OF THE MYELOID-MEDIATED IMMUNOSUPPRESSION MARKER ARGINASE-1 IN CLASSIC HODGKIN LYMPHOMA

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Background: The role of microenvironment in the pathogenesis of classic Hodgkin`s Lymphoma (HL) is well recognized. We previously showed that myeloid-derived suppressor cells (MDSC) have prognostic meaning, but their assay in peripheral blood is barely reproducible.

Aim: Since MDSC are at least in part morphologically and phenotypically similar to neutrophils, we evaluated features and function of mature neutrophils in HL patients (N-HL).

Results: N-HL exhibited a reduced phagocytosis (93.2 ±1.9 % vs 73.1 ± 3.7, p=0.0008) and had an increased arginase expression (at both m-RNA and protein level) and activity up to 15 times compared to healthy subjects. Moreover, in PHA-activated lymphocyte culture from healthy donors, T-cell proliferation and activation markers expression were downregulated by co-culture with HL-N and restored by an Arginase-1 inhibitor. The serum level of Arginase-1 (s-Arg-1) was higher in patients with advanced stage (p=0.045), B-symptoms (p=0.0048) and a positive interim FDG-PETscan after two cycles of chemotherapy (PET-2) (p=0.012). After effective chemotherapy, we observed a reduction of s-Arg-1 within a normal range. Baseline levels of s-Arg-1 >200 ng/mL resulted in 92% sensitivity and 56% specificity to predict a positive PET-2. Patients showing s-Arg-1 levels >200 ng/mL had a shorter progression free survival (PFS). In multivariate analysis, PET-2 and s-Arg-1 at diagnosis were the only statistically significant prognostic variables related to PFS (respectively p=0.0004 and p=0.012). Moving from PET-2 status and s-Arg-1 level we constructed a prognostic score to predict long-term treatment outcome: low s-Arg-1 and negative PET-2 scan (score 0, N=63),with a 3-Y PFS of 89.5%; either positive PET-2 or high s-Arg-1 (score 1, N=46) with 3-Y PFS of 67.6%, and both positive markers (score 2, N=9) with a 3-Y PFS of 37% (p=0.0004).

Conclusions: Neutrophils from HL patients are dysfunctional and show immunosuppressive activity. This inhibition, mediated by s-Arg-1 production, is higher at diagnosis and is related to disease burden and activity. S-Arg-1 can be easily measured in the serum of patients and represents a promising prognostic biomarker either alone and in combination with interim PET.

C036

DASATINIB ENHANCES THE ACTIVITY OF ANTHRACYCLINE-BASED REGIMENS IN PRECLINICAL MODELS OF T CELL LYMPHOMA

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Background: Peripheral T-cell lymphomas (PTCLs) represent approximately 10-15% of all Non-Hodgkin lymphomas (NHL), and their incidence is increasing. Cases of PTCL tend to have an aggressive clinical course, with poor responses to conventional chemotherapy and poor long-term survival. CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) and CHOEP (CHOP+Etoposide) chemotherapy are regimens commonly used despite suboptimal results (Pfreundschuh M, 2014). Alternative approaches as well as new molecular targets are required to ameliorate clinical outcome. Several groups have recently described aberrant tyrosine kinase signaling in PTCL (Agostinelli C, 2014; Netchiporouka E, 2014). Single-agent tyrosine kinase inhibitors (TKIs) have significantly improved patient outcomes across multiple tumor subtypes. However, TKI therapy is rarely curative. Combining TKIs with cytotoxic chemotherapy could represent a new potential therapeutic strategy in PTCL. The present study was designed to test the combination effects of the TKI Dasatinib (Da) and CHOEP in preclinical models of PTCL. Methods: Five T-cell lymphoma and leukemia cell lines (Jurkat, HD-MAR-2, Karpas 299, Sup-T1, HH) were incubated for 48 hours with escalating doses of CHOEP or Da to calculate the IC50 and IC20 values. Analysis of cell viability, cell cycle distribution, apoptosis and mitochondrial depolarization was performed using flow cytometry. ANOVA one-way test was adopted to establish if drug combinations significantly reduced proliferation. Gene expression profiling (GEP) and western blotting (WB) analysis were performed to assess effects of treatments. Results: CHOEP treatment induced concentration and time-dependent growth inhibition in all cell lines, with the most sensitive cells being HH and the least sensitive being HD-MAR-2 (5 fold more resistant). The addition of Da to CHOEP significantly inhibited cell proliferation in SUP-T1, Jurkat, HD-MAR-2 and HH cells (median inhibition: CHOEP 20,5%±5 range 16%-28%; Da 18%±2%, range 16%-21%; Da-CHOEP 52%±12% range 50%-76%; median±SEM, p≤0.001) but not in Karpas 299 cell line (CHOEP 22%±3%; Da 26%±2%; Da-CHOEP 35%±2%, median±SEM, p:ns). The antiproliferative effect of the Da-CHOEP combination was related to a significant increase in cell death (median increase: CHOEP 18%, range:6%-46%; Da 12%, range 4-18%; Da-CHOEP 54%, range 17-93%; p≤0.001) associated with a severe mitochondrial depolarization following caspase-9 activation. GEP and WB analysis showed that CHOEP treatment induces a marked upregulation of tyrosine kinase genes and higher phosphorylation status of intracellular proteins. Src family kinases are primarily phosphorylated and activated in the least sensitive cell lines, thus accounting for the positive action of the combination. Conclusions: The addition of Dasatinib to CHOEP potentiates drug-induced cell death in preclinical models of PTCLs through the dephosphorylation of SRC family kinases.

C037

SEMI-QUANTITATIVE PARAMETERS TO IMPROVE POSITIVE PREDICTIVE VALUE OF INTERIM FDG-PET/CT IN HODGKIN LYMPHOMA

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Introduction: Interim FDG-PET/CT (iPET) after two cycles of chemotherapy ABVD evaluated according to the 5-point Deauville score (5p-DS) has become the strongest predictor for outcome in Hodgkin Lymphoma(HL), and is increasingly used to guide therapy in clinical studies. In PET-guided treatment approaches, iPET-positive patients are candidates for more intensive and potentially more toxic treatments. Nevertheless, recent studies have shown that the positive predictive value (PPV) of iPET visual analysis with 5p-DS has still some limitations to optimally identify patients at different prognosis. Semi-quantitative parameters could improve PPV of iPET and help clinicians to early define patients with worse prognosis. Aim of this retrospective study is to compare PPV of iPET visual and semi-quantitative analysis in patients with HL during first-line chemotherapy with ABVD.

Methods: We studied 67 patients with HL (median age 39 years, range 16-72; 30 females, 37 males) diagnosed at our Institution between 2007 and 2013 and treated with ABVD. Stage was limited in 38 patients and advanced in 29 patients. iPET was performed after 2 cycles of ABVD. For visual analysis, the 5p-DS was used, setting different cut-points at 5p-DS>3 and 5p-DS>4 as positive. We also evaluated interim ratio between lesion and liver SUVmax (rPET); ratio between lesion and mediastinal blood pool SUVmax (mPET); ratio between lesion SUVpeak and liver SUVmean (qPET) as semi-quantitative parameters. Primary endpoint was two-year progression-free survival (PFS), defined as time from date of diagnosis to date of relapse or disease progression. We also valuated i-PET PPV by visual analysis and semi-quantitative parameters. ROC analysis was used to determine the best cut-point of semi-quantitative parameters to identify treatment failures.

Results: In visual analysis, 25/67 patients had 5p-DS >3, and 5p-DS was >4 in 14/67 patients. Two-year PFS according to 5p-DS>3 and 5p-DS>4 was 53% and 27%, respectively. Nevertheless, 5p-DS>3 and 5p-DS>4 had a low PPV (40% and 57%, respectively). The semi-quantitative parameters between residual lesion and the different backgrounds rPET, mPET and qPET were prognostic factors in our population ($p<0.01$). The most accurate cut-point in predicting adverse events for rPET, mPET and qPET were 1.14 (specificity 94.2%, sensitivity 46.6%), 2 (specificity 96.1%, sensitivity 40%) and 1.46 (specificity 96.8%, sensitivity 33.3%), respectively. For values higher than these cut-point, two-year PFS were 15%, 25% and 20%, respectively and the PPV were 70%, 63% and 80%, respectively.

Conclusions: iPET semi-quantitative parameters appear to perform better than visual analysis for outcome prediction in HL. In particular, ratios between residual lesion and background SUV (liver or mediastinal blood pool) could improve the predictive value of relapse or progression.

C038

THE PROGNOSTIC ROLE OF FDG-PET/CT AT THE END OF INDUCTION IMMUNOCHEMOTHERAPY (PI-PET) IN PATIENTS WITH FOLLICULAR LYMPHOMA: A RETROSPECTIVE MONO-CENTRIC STUDY

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Although the outcome of patients (pts) with Follicular Lymphoma (FL) has clearly improved, heterogeneous behavior of the disease still remains. Currently, FLIPI is the most widely used prognostic systems, but none of the current available prognostic factors has been used to guide clinical decisions in FL. 18F-fluorodeoxyglucose-positron emission tomography/computed tomography (FDG-PET/CT) was recently shown to add details in the staging and to be an independent prognostic factor for lymphoma progression when used at the end of induction immunochemotherapy (ICHT) in pts with FL (PI-PET). Several visual system to evaluate FDG-PET in FL have been proposed, such as 2007 International Harmonization Project (IHP) and 5-point Deauville Score (5p-DS). The aim of our study was to investigate the prognostic role of FDG-PET/CT scan at the end of ICHT (PI-PET) in pts with FL. We retrospectively analyzed 105 pts with high-tumor burden FL diagnosed between 2004 and 2014 in our Institution and who received systemic ICHT (R-CHOP, R-CVP, R-Bendamustine and fludarabine containing regimens). All pts were evaluated with a PI-PET at the end of the treatment. Firstly, IHP criteria was used to classify scans as positive or negative. Fisher's exact test was used to correlate PI-PET and pts characteristics at diagnosis. PI-PET results were correlated with progression-free survival (PFS) and overall survival (OS). Fifty pts (50/105, 48%) continued maintenance therapy with Rituximab. Finally, PI-PET evaluation according to DS criteria (score 0-3 negative, 4-5 positive) was retrospectively applied on 43/105 (41%) pts. According to IHP criteria, PI-PET was positive in 25/105 (24%) pts, and negative in 80/105 (76%) pts. PI-PET positivity was significantly associated to initial bulky disease ($p=0.03$), and to number of nodal sites ($p=0.07$). Pts with a positive PI-PET had significantly lower 5 year PFS (52%, 95% C.I. 28-72 %) compared to pts with a negative PI-PET (85%, 95% C.I. 72–92 %, $p= 0.003$). Restricting the analysis to pts who continued maintenance therapy, 15/50 (30%) pts had a positive PI-PET, pts, while 35/50 (70%) pts had negative PI-PET. Pts with a positive PI-PET still had a significantly lower 5 year PFS (52%, 95% C.I. 21-75%) when compared to pts with a negative PI-PET (100%, $p= 0.0003$) despite maintenance therapy. Evaluating the PI-PET according to 5p- DS criteria, only 6/43 (14%) pts had positive PI-PET, while it was negative in 39/43 (86%). Pts with a positive PI-PET according to5p-DS had significantly lower 3 years PFS (40%, 95% C.I. 0.01-0.83) compared to pts with negative DS PI-PET (89%, 95% C.I. 0.64-0.97, $p=0.005$). We conclude that PI-PET is a strong prognostic factor, and maintains its prognostic power also in pts who continue maintenance therapy. DS might be a better predictor of early progression than IHP criteria. Finally, PI-PET could be useful to identify pts with FL at risk for early relapse after ICHT.

C039

IDENTIFICATION OF GENE EXPRESSION PROFILES ASSOCIATED TO SENSITIVITY AND RESISTANCE TO BENDAMUSTINE (BDM) AND TO THE 'FIRST-IN-CLASS' ALKYLATING HDACI FUSION MOLECULE EDO-S101 IN TUMOR CELLS OF HODGKIN LYMPHOMA

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Introduction. Development of active agents for patients with Hodgkin lymphoma (HL) who progress after SCT is an impellent medical need. BDM is active in recurrent HL but some patients fail or become resistant to the drug. While mechanisms underlying resistance to BDM in HL cells are unknown, inhibitors (i) of the HDAC complex may amplify alkylators-induced DNA damage by deranging DNA repair pathway. This concept led to design of EDO-S101, a hybrid molecule fusing the BDM alkylating mojety with a pan-HDACi (vorinostat). We analyzed changes in gene expression elicited by EDO-S101 in BDM-sensitive and -resistant L1236 HL cells.

Methods. L1236 cells underwent RNA-sequencing (NGS technology; human reference genome GRCh37/hg19) before and after a 48h exposure to BDM (50 uM) and EDO-S101 (5 uM). RNA was also obtained after exposure to EDO-S101 of R100 cells, a BDM-resistant L1236-derived cell line. Differentially expressed genes (BDM- and EDO-S101-exposed vs untreated L1236 cells; EDO-S101-exposed vs untreated R100 cells) were analyzed and data on the involved molecular pathways obtained by the GeneCards¬Æ-The Human Gene Database software.

Results. RNA-sequencing identified 28780 transcripts with a different constitutive expression in L1236 cells. Upon exposure to BDM and EDO-S101 levels of 259 and 382 transcripts were respectively modified. Some gene-sets were synchronously upregulated by both agents. These involved cell growth- and HL-specific pathways (TNFRSF8/CD30, TNF, PI3KR5, CDNK1A, CCL17-2-3, CCR4, CXCR3, IL13-6, SMAD7, GDF15) and AKT/PI3K signaling. Some apoptosis genes (CASP1, CASP4, CARD16) were synchronously turned off, while EDO-S101 asynchronously increased 29 transcripts encoding cell stress products (PAG1), proapototic effectors (ISM1, PKB), transcriptional regulators (TFEC), tumor suppressors (INHBA) and p21-activators (PAK3). Genes (n=24) involved in drug-resistance and migration/invasion (GAGS2, ANO9, MGLL) were turned off. R100 cells showed i) upregulation of chemokine genes (CCL3-22-17), genes involved cell growth (TNFRSF8-4-1a-10c, NGFR, IL6,-4,-31, LYN, FYN, RAP2A), cell adhesion and tumor promotion (ICAM3, glipican6, paxillin, FXYD5). This was coupled to a massive shut down of genes ensuring the proper functioning of the MHC-Class II machinery. Exposure to EDO-S101 of R100 cells downregulated 21 genes involved in DNA repair and PI3K/AKT signaling, while transcripts for 14 genes, including the inhibitory MIR17HG, were upregulated.

Conclusions. Results of NGS data support that EDO-S101, beyond sharing with BDM a common gene-regulatory profile in L1236 cells, may activate a specific gene pattern leading to cell death in both L1236 and R100 cells. Data also suggest that exposure to both agents triggers in HL cells a 'survival response' amplifying their capability to gain pro-survival signaling from microenvironment and activate pathways to escape immune surveillance. This 'response' can guide the design of combination treatments.

C040

SENSITIVITY OF ULTRASOUND-GUIDED 16 G CORE-NEEDLE CUTTING BIOPSY AND EXCISIONAL BIOPSY FOR THE CHARACTERIZATION OF LYMPHADENOPATHIES IN PATIENTS WITH SUSPECTED LYMPHOMA: A RANDOMIZED SUPERIORITY TRIAL

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Background: Histological examination of adequate biopsy specimens is essential for lymphoma diagnosis. Although core-needle cutting biopsy (CNCB) under image guidance offers an alternative to open biopsies, its sensitivity in lymphoma diagnosis is still a matter of debate. The introduction of new generation ultrasonographic (US) devices and progress in tissue sampling with needles with cutting edge of adequate diameter have enabled to obtain enough tissue for a definitive diagnosis by mini-invasive procedures.

Aims: The primary endpoint was to demonstrate the superiority in terms of sensitivity in detecting malignancy of US-guided CNCB compared with standard excisional biopsy of suspected lymphoadenopathies. Secondary endpoints were: specificity, positive predictive value (PPV), negative predictive value (NPV), complication rate and cost analysis.

Methods: From January 2009 to December 2015, in this single centre trial, patients having lymphoadenopathies with clinical suspicion of lymphoma were randomly assigned (1:1) to either US-guided CNCB or standard excisional biopsy. In the US-guided group, patients underwent baseline US exploration of all superficial lymph node areas and any abnormal lymph node underwent power-Doppler study to select the site of CNCB. The CNCB were all performed using a 16 gauge modified Menghini-type needle 150 mm in length with automatic aspiration (Biomol HS-Hospital). The selection of lymph node in the excisional biopsy (standard) group was suggested by the physical examination.

Results: Overall, 372 patients were randomized into two arms. Histology showed a malignancies in 93% (172/185) of patients in the US-guided group (lymphoma, 151 patients; carcinoma, 21 patients) and in 80% (149/187) of patients in the standard group (lymphoma, 122 patients; carcinoma, 27 patients). During the follow-up of the patients with lymph nodes reported as reactive, 19 of 38 patients in the standard group were rebiopsied and were found to have malignancies, whereas three patients in the US-guided group requiring a second biopsy were found to be positive for lymphoma. Thus, sensitivity in detecting malignancy was higher in the US-guided group compared with the standard group (172/175 [98%] vs. 149/168 [89%]; p=.0003), demonstrating the superiority of US-guided CNCB. Biopsy provided false-negative results in 10.2% of patients in the standard group and 1.6% in the US-guided group (p=.0008). No differences were found in terms of specificity, NPV and PPV between the two diagnostic techniques. Estimated cost per diagnosed with traditional biopsy was 20-fold higher compared with US-guided CNB group (p<.0001). Mean complication rate was significantly higher in the standard group (40.5%) than in US-guided group (18.7%), p<.0001.

Conclusions: US-guided CNCB has proven to be a quick, safe, and efficient technique and has radically altered the diagnostic strategy of enlarged lymph nodes at our institution, avoiding unnecessary lymph node excisions.

Stem Cells and Growth Factors

C041

BONE MARROW ADIPOCYTES SUPPORT THE SURVIVAL AND THE DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS

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Introduction: Bone marrow adipocytes (BM-A) represent the most abundant stromal component of BM and together with other stromal population they play an active role within the marrow, regulating hematopoiesis through cell-to-cell contact and secreting growth factors and cytokine. With ageing their number gradually increase and they may occupy up to 50% of BM cavity replacing the BM hematopoietic component. The substitution of red hematopoietic BM with fatty yellow BM suggests an inverse relationship between adipocytes amount and BM hematopoietic activity. However it has also been demonstrated that BM-A express MSC and HSC markers and produce cytokines and adipokines directly involved in hematopoiesis regulation. This scenario suggests that the role of fat in BM microenvironment is still largely unknown, therefore in this work we functionally and molecularly characterized BM-A, studying more in detail their role in hematopoiesis.

Methods: BM-A were isolated from the head of a femur of patients undergoing hip surgery after collagenase digestion and filtration, we studied their morphology and their transcriptome through microarray technology comparing them to adipose tissue adipocytes (AT-A). BM-A functional properties in the hematopoietic microenvironment were assessed studying the secretion of cytokines and the hematopoietic supporting capabilities through LTC-IC culture, comparing them with BM-MSC.

Results:In this work we demonstrated that BM-A are able to support the maintenance and the differentiation of hematopoietic progenitor cells. More in detail BM-A constituted the feeder layer of $CD34^+$ normal cells in culture for 5 weeks and when $CD34^+$ cells were detached and seeded into a specific methylcellulose medium, they were still able to proliferate and differentiate forming CFU-GM and BFU-E. CFU-GM and BFU-E staining showed that most of cells were represented by macrophages but also granulocytes and myelocytes were visible. Few erithroyd lineage cells have been observed. We also demonstrated that BM-A displayed a completely different gene expression profile respect to AT-A showing different lipid metabolism, stem cell gene and white and brown differentiation pathways regulation. Also hematopoietic cell-to-cell signaling pathways and secreted factors analysis showed that BM-A are closely related to BM-MSC demonstrating a positive involvement of these cells in hematopoiesis support and regulation. Cytokines secretion was further evaluated through cell culture supernatant analysis showing that critical molecules as CXCL12, IL3 IL8, CXCL-12, G-CSF and LIF, were expressed at similar level in BM-A and BM-MSC while IL3 is even higher expressed in BM-A.

Conclusions: Our results not only support the hypothesis that BM-A play an active role in BM microenvironment but strongly suggest that BM-A can provide a supporting role for the hematopoietic niche directly sustaining the survival, the proliferation and the differentiation of HSC.

C042

HUMAN WHITE ADIPOCYTES CONVERT INTO "RAINBOW" ADIPOCYTES *IN VITRO*

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Introduction: White human adipose tissue adipocytes (AT-A) are plastic cells able to reversibly transdifferentiate into brown adipocytes and into epithelial glandular cells under physiologic stimuli *in vivo*. These plastic properties could be used in future for regenerative medicine, but are incompletely explored in their details. Here, we studied the plastic properties of AT-A either isolated or cultivated by primary ceiling culture combining gene expression profile analysis with morphological data analysis with morphologic data obtained by electron and time lapse microscopy. We also documented the secretion of large lipid droplets process occurring *in vitro*, which represent the main morphologic aspect of the transdifferentiation process through electron and time lapse microscopy.

Methods: AT-A were isolated from donors biopsies at the time of abdominal surgery after collagenase digestion and filtration. Transcriptome analysis was assessed through microarray analysis and gene expression level were further evaluated through real time PCR. Time lapse and electron microscopy were used to document the transdifferentiation process.

Results: Primary MA showed the classic morphology and gene expression profile of functional adipocytes, with up-regulation of genes involved in lipid metabolism and down-regulation of those responsible for cell shape changes and proliferation. Notably, despite their committed status, MA expressed high levels of stem cell and reprogramming genes. MA from ceiling cultures underwent transdifferentiation towards fibroblast-like cells with a well-differentiated morphology and morphological analysis showed that this process is not due to a progressive loss of lipids such as it happens during fasting-induced lipolysis but that it is rather due to massive liposecretion. We observed that a distinct trilaminar plasma membrane progressively surrounded the lipid droplet vacuoles. After 4-6 days in culture, adipocytes acquired a more elongated shape. Most of the lipid vacuoles were surrounded by a distinct trilaminar plasma membrane at this stage, and cytoplasmic gaps observed strongly suggested a process of liposecretion. The *in vitro* liposecretion hypothesis was further confirmed by time-lapse microscopy and images of lipid droplet secretion from MA were acquired during 50 hours. The subsequent 20 time-lapse hours demonstrated the viability of post secretion adipocytes showing cellular motility with BM-MSC-like structure (Video).

Conclusions: In conclusion, isolated human adipocytes share many phenotypic aspects with other well-recognized stem cells. In primary culture they convert through a transdifferentiation process that involves liposecretion into well-differentiated fibroblast-like cells that maintain the high plastic propensity of mature adipocytes. These data support the idea that mature adipocytes can be used as stem cells in regenerative medicine.

C043

MIR-382-5P REGULATES MEGAKARYOCYTE AND GRANULOCYTE COMMITMENT BY TARGETING MXD1

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Introduction. microRNAs (miRNAs) are key regulators that control stem cell fate by post-transcriptionally down-regulation of hundreds of target genes via seed-pairing in their 3' untranslated region. Networks of transcription factors and miRNAs tightly regulate commitment of hematopoietic stem cells along the different lineages and miRNA deregulation may contribute to the development of hematological malignancies. In this study, we focused our attention on miR-382-5p, which has been reported to be overexpressed in Acute Myeloid Leukemia (AML) samples with t(15;17) (Li Z et al. PNAS 2008) and in Primary Myelofibrosis (PMF) patients (Norfo R et al. Blood 2014). Despite the abnormal expression of miR-382-5p described in leukemogenesis, its role in normal hematopoiesis has not been described so far.

Methods: In order to study the role of miR-382-5p in myeloid lineage, CD34+ cells were transfected with miR-382-5p mimic by using the 4D-Nucleofector System. Clonogenic and liquid culture assays were performed to evaluate the effects of miR-382-5p overexpression. Furthermore, in order to identify its molecular targets, two different prediction algorithms (TargetScan and microRNA.org) were used and selected putative targets were validated by luciferase reporter assay.

Results: Flow cytometric analysis showed that miR-382-5p overexpression induces a significant decrease in the CD41+ fraction coupled with a relevant increase of CD15+ and CD66b+ cells. According to these results, the morphological analysis of MGG-stained cytospins showed a remarkable reduction in megakaryocyte (MK) precursors at different stages of maturation and a considerable enrichment in granulocytes (GN) in miR-382-5p-overexpressing cells compared with control. Moreover, methylcellulose assay highlighted a strong increase in the percentage of granulocyte colony forming unit (CFU-G), whereas monocyte (CFU-M), erythroid (Burst-Forming Units (BFU-E) and CFU-E) and granulomonocyte (CFU-GM) colonies were not significantly affected. Moreover, the collagen-based assay supporting the growth of MK progenitors *in vitro* showed that miR-382-5p overexpression causes a significant decrease in the CFU-MK percentage. Target prediction analysis identified several putative targets of miR-382-5p, which are known to be involved in hematopoiesis (i.e. GATA2, FLI1, RUNX1, MXD1, PTEN, MAF and SGK1). The expression of all these genes was decreased in miR-382-5p-overexpressing cells. Therefore, we tested every putative miR-382-5p/target pair by luciferase reporter assays. Among these, we were able to validate only MXD1. Finally, as observed for miR-382-5p overexpression, MXD1 knockdown in CD34+ cells led to significant decrease of MK precursor coupled to increase of GN lineage.

Conclusions: Overall, these results indicated that miR-382-5p favours GN commitment at the expense of MK lineage by means of MXD1 down-regulation.

C044

DIFFERENTIAL AND TRANSFERABLE MODULATORY EFFECTS OF MESENCHYMAL STROMAL CELL-DERIVED EXTRACELLULAR VESICLES ON T, B AND NK CELL FUNCTIONS

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Mesenchymal stromal cells (MSCs) are multipotent cells, immunomodulatory stem cells that are currently used for regenerative medicine and treatment of a number of inflammatory diseases,thanks to their ability to significantly influence tissue microenvironment through the secretion of large variety of soluble factors. Recently, several groups have reported the presence of extracellular vesicles (EVs) within MSC secretoma, showing their beneficial effect in different animal models of disease. Here, we used a standardized methodological approach to dissect the immunomodulatory effects exerted by MSC-derived EVs on unfractionated peripheral blood mononuclear cells andpurified T, B and NK cells.

We describe here for the first time a direct correlation between the degree of EV-mediated immunosuppression and EV uptake by immune effector cells, a phenomenon further amplified following MSC priming with inflammatory cytokines. Accordingly, B cells were mostly prone to incorporate EVs and to be modulated, as compared to other lymphocyte subsets; similarly, EV suppressive potential towards NK and T cell proliferation was proportional to their uptake by those cell types. Despite MSC-derived EVs did not inhibit directly T cell proliferation, when resting MSCs were activated with primed EVs showed a significant increase in their immunosuppressive properties, an effect observed only in presence of T cells. This phenomenon suggested an indirect effect of MSC-derived EVs towards T cell proliferation, a mechanism that would appear to be mediated by IDO increase. Moreover, miRNA-155 and miRNA-146, which have an important role in the modulation of immune response, were detected inside EVs delivered by MSCs. Interestingly, MSC licensing triggered this mechanism: following cytokine activation, MSCs significantly enhanced the expression and the release of these immunomodulatory molecules within EVs. These data suggest a hypothetical involvement of EV-derived miRNAs both in the direct effect of EVs on IEC proliferation and in their capability to increase MSC priming.

The *in vitro* immunological features of MSC-derived EVs here described are consistent with their beneficial effects observed *in vivo* in different inflammatory diseases, including refractory GvHD, severe refractory acute respiratory distress syndrome (ARDS) and acute kidney injury. The *in vivo* administration of MSC-derived EVs could reduce inflammation inside damaged organs and make resident MSCs immune regulatory cells.

Our study provides new evidence on the immunological activity of MSC-derived EVs towards different IECs and the usefulness of quantitative and reproducible immunological assays to characterize MSC-derived EV immunomodulation. However, *in vivo* studies are mandatory to evaluate the potential clinical benefit of using EVs rather than ex-vivo expanded, clinical grade MSCs.

C045

PENTRAXIN 3 PLASMA LEVELS AT GRAFT-VERSUS-HOST DISEASE ONSET PREDICT DISEASE SEVERITY AND RESPONSE TO THERAPY IN CHILDREN GIVEN HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Introduction: Acute Graft-versus-Host Disease (GvHD) is a major complication of allogeneic hematopoietic stem cell transplantation (HSCT). Systemic corticosteroids, administered as first-line treatment, are only partially effective. Reliable biomarkers predicting disease severity and response to treatment are currently limited. We sought to determine whether pentraxin 3 (PTX3), an acute-phase protein produced locally at the site of inflammation, could be used as a novel biomarker for the diagnosis and management of acute GvHD.

Methods: PTX3 plasma levels were evaluated in a fully MHC-mismatched mouse model of acute GvHD. PTX3 levels were further evaluated in 115 pediatric HSCT patients with hemato-oncological diseases (S. Gerardo Hospital, Monza and at the Regina Margherita Hospital, Turin). Plasma samples were collected before conditioning regimen (basal), at HSCT, weekly until day 100, at GvHD onset. PTX3 was also evaluated by immunohistochemistry in GvHD lesions. To investigate its role in GvHD pathogenesis, rhPTX3 was injected in allo-transplanted mice.

Results: In the mouse model, PTX3 levels, low before irradiation increased 8-folds, as effect of conditioning regimen. Thereafter, the protein decreased in both syngeneic and allogeneic transplanted mice, before raising again at GvHD onset only in the allogenic group (p=0.03). In the pediatric cohort of HSCT patients we observed a significant increase of PTX3 from a basal of 7.19 up to 31.39 ng/ml at day 0, as effect of conditioning. PTX3 levels were next compared in patients experiencing or not the disease within 100 days after HSCT. At the onset of GvHD, PTX3 resulted significantly higher than in time-matched plasma samples of the No GvHD group. Evidence of increased PTX3 production was also found in skin and colon biopsies from patients with active disease. To evaluate the predictive role of PTX3, we firstly correlated protein levels at disease onset with GvHD severity. PTX3 resulted significantly increased in patients with severe GvHD, compared to patients with milder disease. We next correlated PTX3 levels with therapy response. Interestingly, PTX3 resulted 3-fold higher at disease onset in patients who had no response after 4 weeks, compared to patients who experienced a complete or partial response. To assess whether, besides representing a GvHD biomarker, it could also play a role in disease pathogenesis, we injected recombinant human PTX3 in our mouse model of GvHD. No differences were observed between animals treated or not with the molecule in terms of overall GvHD score and histopathology on GvHD lesions, thus ruling out a direct effect of the molecule on disease course.

Conclusions: Our results candidate PTX3 as a reliable biomarker reflecting GvHD severity and therapy responsiveness. If these results will be confirmed in a larger cohort of patients, PTX3 could represent a clinically relevant tool for tailoring patient-specific anti-GvHD therapy soon as the pathology occurs.

C046

THE CHEMERIN/CHEMR23 AXIS PLAYS A PIVOTAL ROLE IN THE PATHOGENESIS OF INTESTINAL DAMAGE IN A MURINE MODEL OF GRAFT VERSUS HOST DISEASE

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Introduction: Graft-versus-Host Disease (GvHD), represents a major complication after Hematopoietic Stem Cells Transplantation. Chemerin has been recently identified as a chemotactic protein, which is produced by several tissues during inflammation and binds the G protein-coupled receptor ChemR23, expressed by DCs, macrophages and NK cells. The aim of this study was to evaluate the potential role of Chemerin/ChemR23 axis in GvHD pathogenesis, in order to identify disease-specific pathways exploitable for developing new potential therapeutic targets.

Methods: Lethally irradiated Balb/C recipient mice were transplanted with bone marrow cells and splenocytes obtained from ChemR23-deficient C57BL6 mice (tChemR23-KO). Mice were monitored daily for survival and GvHD severity. Recipient mice were sacrificed at different time points to evaluate Chemerin production and leukocytes infiltration in GvHD target organs.

Results: Starting from day +6 after transplantation, Chemerin plasma levels appeared significantly higher in both wild type (WT) and tChemR23-KO mice who developed GvHD, compared to syngeneic controls. Interestingly, tChemR23-KO mice developed a more severe GvHD compared to mice transplanted with WT cells. In particular, tChemR23-KO mice showed a higher mortality rate. Differences in GvHD score between ChemR23-KO and WT transplanted mice resulted by a significantly increase in weight loss, associated to severe diarrhea. In accordance, histological analysis performed on GvHD target organs showed a significantly higher GvHD score in large intestine of tChemR23-KO mice, whereas no differences were found in other GvHD organs. In addition, a deeper histological analysis on large intestine showed that tissue damage is characterized by crypt hyperplasia and atrophy, epithelium apoptosis and colitis. FACS analysis of large intestine infiltrating leukocytes showed that the percentage of neutrophils infiltrating colon were significantly higher in tChemR23-KO mice compared to WT transplanted mice. The higher neutrophils infiltration was also confirmed by immunohistochemistry and RQ-PCR. Interestingly, the analysis of ChemR23+ cell subsets revealed that macrophages infiltrating colon mucosa were significantly lower in tChemR23-KO mice compared to WT, while no differences were observed in DCs or NK cells. All these observations were also obtained by analyzing the mesenteric lymphnodes. The adoptive transfer of WT-monocytes into tChemR23-KO mice, demonstrated that WT-monocytes were able to improve GvHD in terms of survival, weight loss and overall score, thus confirming that lacking of ChemR23 expression on macrophages induces an increase of GvHD damage.

Conclusions: All these findings suggest that the Chemerin/ChemR23 axis plays a crucial role in intestinal GVHD, driving macrophages infiltration in colon mucosa. Further studies are need-

ed to better understand the mechanisms underling the severe damage observed in the large intestine of tChemR23-KO mice.

C047

MOBILIZED PERIPHERAL BLOOD VERSUS CORD BLOOD: DISTINCT ROLE OF PRO-INFLAMMATORY CYTOKINES ON SURVIVAL, CLONOGENIC ABILITY AND MIGRATION OF CD34+ CELLS

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Introduction: The inflammatory microenvironment may modulate the behaviour of the hemopoietic stem/progenitor cells (HSPCs) which actively sense pro-inflammatory factors. The purpose of this study was to compare the survival/function of CD34+ cells derived from umbilical cord blood (CB) and G-CSF mobilized peripheral blood (mPB) after *in vitro* exposure to crucial factors of the inflammatory microenvironment (Interleukin-1 β (IL-1β), Tumor Necrosis Factor (TNF)-α, Tissue Inhibitor of Metalloproteinases-1 (TIMP-1).

Methods: Immunomagnetically isolated CD34+ cells from CB (5 cases) or mPB (4 cases) from hemopoietic stem cells transplantation donors were in vitro incubated at 37°C for 4 days with or without IL-1β (1 ng/mL), TNF-α (10 ng/mL) or TIMP-1 (100 ng/mL), alone or in combination, and cell survival (AnnexinV/PI staining) was assessed. Clonogenic assay (colony forming unit (CFU-C) growth) and in vitro migration towards a C-X-C motif chemokine 12 (CXCL12) gradient in the presence or absence of the pro-inflammatory factors were also performed.

Results: We found that the survival of untreated CB-derived CD34+ cells was significantly increased as compared with the mPB counterparts. When pro-inflammatory factors alone were tested, no significant differences were observed in survival of CB- or mPB-derived CD34+ cells. By contrast, the combination of IL-1β plus TNF-α increased the survival of CB-derived CD34+ cells but did not affect that of mPB-derived CD34+ cells. Notably, the survival of CB CD34+ was significantly promoted by the addition of TIMP-1 to IL-1β+TNF-α as compared to a slightly promotion in mPB. Pro-inflammatory factors, alone or in combination, were ineffective in stimulating the CFU-C growth in CB- or mPB-derived CD34+ cells. However, the combination of IL-1β, TNF-α, TIMP-1 increased the CB-derived CFU-C growth as compared with untreated and mPB-derived cells. Interestingly, when BFU-E and CFU-GM growth were individually analysed, the results showed that IL-1β alone promoted the BFU-E growth of the mPB-derived CD34+ cells. In addition, IL-1β+TNF-α+TIMP-1 significantly augmented the numbers of BFU-E and CFU-GM in CB-derived CD34+ cells when compared with mPB-derived cells. Finally, only the migration of CB-derived cells was significantly promoted by factors alone and IL1β+TIMP-1 as compared to untreated samples. Nevertheless, unlike CB counterparts, the combination of IL-1β, TNF-α and TIMP-1 enhanced the migration of mPB-derived CD34+ cells.

Conclusions: Our results demonstrate a distinct sensitivity of CB and mPB cells to the inflammatory microenvironment. Together these findings emphasize that CB- and mPB-derived CD34+ cells show *in vitro* different functional response to selected pro-inflammatory factors.

C048

DIFFERENT COMPOSITION OF HEMATOPOIETIC STEM AND PROGENITOR CELLS BETWEEN PERIPHERAL BLOOD AND BONE MARROW: BIOLOGICAL AND CLINICAL IMPLICATIONS

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In the last years, peripheral blood (PB) hematopoietic stem and progenitor cells (HSPC) have been widely used for hematopoietic stem cell transplantation (HSCT) procedures. One of the main advantage instead of using bone marrow (BM) HSPC consists in a more rapid hematologic and immunological recovery. Oppositely to BM, very few is known about PB HSPC constitution. In this project, we intend to analyze the ratios of peripheral HSPC in order to see whether the numbers of re-infused HSPC could influence kinetic engraftment after an HSCT.

Multicolor flowcytometry was run to examine lineage negative (Lin-) CD34+ cells from 9 PB and 4 BM samples from healthy donors and 32 mobilized PB (mPB) samples from hematological patients prior CD34+ cells harvesting.

On healthy donors, common myeloid progenitors (CMP) were 47.8% in PB versus 27.6% in BM ($p<0.005$); on the contrary, granulocyte-macrophage progenitors (GMP) were lower in PB (10.3%) compared to BM (23.8%, $p<0.01$). No differences were displayed between PB and BM hematopoietic stem cells (HSC). On G-CSF mobilized Lin- CD34+ cells, GMP were 17.7% while in non mobilized samples just 9.6% ($p=0.04$); megakaryocyte-erythrocyte progenitors (MEP), instead, were 8.6% mPB versus 14.8% in non mobilized PB ($p=0.02$). We then analyzed percentages of subpopulations on 2 Plerixafor, the CXCR4 inhibitor, treated patients: interestingly, GMP showed a critical higher ratio (37.8% and 33.8%) compared to the G-CSF-only mobilized samples (average of 16.3%). Looking then at CXCR4 expression on all Lin- CD34+ cells, we demonstrated a higher mean fluorescence intensity on GMP compared to the other subpopulations ($p=0.0001$).

The review of clinical data showed a significant correlation between the total number of peripheral CD34+ cells and the amount of mobilized CMP ($p<0.001$), GMP ($p<0.001$) and MEP ($p<0.001$), but not with mobilized HSC, that showed, instead, a correlation with the total WBC count ($p<0.01$). Regarding the kinetic of engraftment after an autologous HSCT, we saw a tendency to inverse correlation among the number of days with neutrophil count < 500/uL and the quote of re-infused CMP, GMP and MEP and among platelets levels in the first 15 days after the procedure and re-infused CMP and MEP.

In conclusion, in our study we demonstrated the great heterogeneity of HSPC composition between PB, BM and mPB. Important roles are played by CMP and GMP; GMP in particular are strongly influenced by chemo (data not shown) and mobilization regimens. It is known that GMP are the expanded clonal population among the majority of AML patients (Goardon N, Cancer Cell, 2011). Therefore, our data suggest the potential role of Plerixafor combined with traditional chemotherapies on AML and its absolute contraindication on mobilizing regimens for autologous transplantation on AML patients. Further studies with higher sample sizes are required to validate the correlation between number of re-infused HSPC and engraftment data.

Immunotherapy and cell therapy

C049

CHEMOTHERAPY-DEPENDENT ATP RELASE FROM LEUKEMIA DYING CELLS INDUCES INDOLEAMINE 2,3-DIOXYGENASE IN DENDRITIC CELLS

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Introduction: Recently, both in solid tumors and haematological malignancies, it has been shown that some chemotherapeutic agents, such as daunorubicin (DNR), are highly immunogenic and activate the immune response by the induction of immunogenic cell death (ICD). Such process is characterized by modifications and emission of damage signals from dying cells which are detected by dendritic cells (DCs), thus eliciting immune system activation. Among these signals, ATP is a key mediator of immunogenicity during ICD. However, along with ICD, chemotherapy is known to induce inflammatory modifications within tumor microenvironment, which may also elicit immunosuppressive pathways. In particular, the expression of the tolerogenic enzyme indoleamine 2,3-dioxygenase (IDO1) may be induced in DCs, which may hamper anti-tumor immune response.

Methods: *In vitro*, murine WEHI-3B and human HL-60 leukemic cell lines and primary blasts from patients were exposed to DNR and tested for ATP release. To investigate *in vivo* DNR-induced ICD, WEHI-3B cells stable transfected with luciferase PmeLUC were inoculated subcutaneously in BALB/c mice and used to measure ATP release from tumor mass using a total body luminometer. Tumor infiltrating DCs and T cells were characterized by FACS analysis and immunohistochemistry. *in vitro*, DNR-treated AML cells were pulsed into immature DCs, previously generated from healthy donors. Then, their maturation status and IDO1 expression was examined by FACS and western blot respectively and correlated with the presence of ATP in culture medium. IDO-driven Tregs induction was also assessed. Finally, functional immunological tests were performed *in vitro* to test the ability of Tregs to inhibit leukemia antigen-specific IFN-gamma production (FACS analysis) by ICD-activated T cells.

Results: DNR treatment increased ATP release from AML cells *in vitro* and *in vivo*. In DNR-treated mice we observed a significant increase of CD11c+ mature DCs which express IDO1 in tumor infiltrate. *in vitro*, loading of DNR-treated AML cells into DCs resulted in increased maturation, but also in IDO1 induction, which is involved in Tregs expansion. Interestingly, we showed that extracellular ATP is directly involved in DCs maturation and IDO1 expression via P2Y11 receptor. As expected, ICD-driven DCs were able to expand a population of Tregs in an IDO-dependent manner. Finally, we found that ICD triggers a leukemia-specific IFN-gamma production by CD8+ T cells but concomitantly induces Tregs, via IDO1-expressing DCs, which were able to reduce anti-leukemia immune response of activated T cells.

Conclusions: Chemotherapy-dependent ATP release from leukemia dying cells induced maturation of DCs which efficiently activate antigen-specific T lymphocytes but simultaneously acquire tolerogenic function, depending on their expression of IDO. The combination of chemotherapy with IDO1 inhibitors may represent an interesting approach to enhance anti-leukemic immune response.

C050

ANTHRACYCLINE-BASED CHEMOTHERAPY FOR AML INDUCES LEUKEMIA-SPECIFIC EFFECTOR T CELLS WITH DEFECTIVE FUNCTION AND EXHAUSTED PHENOTYPE

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Introduction: mmunogenic cell death (ICD) induced by anthracyclines, such as daunorubicin (DNR) is highly immunogenic and results in efficient cross-priming of anti-tumour T cells through dendritic cells . Recently, some reports indicated that anticancer drugs, while triggering ICD, induce also expansion of regulatory T cells (Tregs) in leukemias. In AML, although a good fraction of patients respond to first-line anthracycline-based chemotherapy, a minimal residual disease persists and leads to relapse if not eradicated. A better characterization of activated T cells emerging after chemotherapy in AML patients can help to develop novel immunological therapeutical strategies against the leukemic clones, which are not sensitive to the cytotoxic effect of chemotherapy. METHODS: T cells of 27 AML patients undergoing DNR-based induction chemotherapy, were analyzed. Before and at different time points after chemotherapy (+7, +14, +21 and +28), the frequency of IFN-γ producing CD4+ and CD8+ T cells and circulating Tregs were analysed by FACS. Then, CD3+ T cells after chemotherapy were isolated from AML patients, catched for IFN-γ, TNF-α, 4-1BB and CD8 and sorted After expansion, CD8+ T cells were phenotypically and functionally characterized. In particular, cytotoxicity and cytokines production were evaluated. In mouse model, mice, subcutaneously injected with AML murine cell line WEHI-3B, were treated with DNR or placebo and analyzed for plasma levels cytokines. Moreover, tumour-infiltrating T cells were phenotypically characterized. RESULTS: AML cells treated with DNR undergo ICD mechanisms, including CRT and HSPs70 and 90 translocation and release of ATP and HMGB1. 18/27 AML patients, undergoing DNR-based chemotherapy, have an increase in leukemia-specific IFN-γ producing CD4+ and CD8+ T cells, mainly at day 14 and 21 after chemotherapy. Similarly, an increase of Tregs was observed with a peak at day 21. CD8+ IFN-γ-producing T cells showed an exhausted phenotype represented by down-regulation of CD28 and up-regulation of Lag-3 and PD-1, in particular in effector memory and effector memory expressing RA T-cell subpopulations. Accordingly, the granzyme B-mediated cytotoxicity against autologous blasts was observed only at elevated CTL/target cell ratio. In the mouse model, DNR treatment increased plasma levels of activatory (IFN-γ, IL-1β, TNF-α, IL-12) and tolerogenic (IL-10) cytokines. Interestingly, tumour-infiltrating CD8+ T cells after DNR treatment showed an exhausted phenotype, increase in IFN-γ-producing Tregs and decrease in TNF-α,-producing T effector cells.

Conclusions: These *ex vivo* and *in vivo* data demonstrate that anthracycline-based chemotherapy in AML is highly efficient in eliciting leukemia-specific T cells through ICD, but also induces an exhausted phenotype of CD8+IFNγ+ T cells which demonstrated limited cytotoxic potential. These data indicate the need for further characterizing the tolerogenic pathways, developing along with ICD.

C051

CD4+FOXP3+ REGULATORY T CELLS MAINTAIN THE BONE MARROW MICROENVIRONMENT, PROMOTE B CELL DIFFERENTIATION AND INDUCE TOLERANCE TO BONE MARROW GRAFTS

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Introduction: CD4+FoxP3+ regulatory T cell (Treg) adoptive transfer has proved to prevent GVHD while Treg impact on immune reconstitution and engraftment has been less well studied.

Methods: We tested the impact of Diphtheria Toxin (DT) induced Treg ablation in C57BL/6 FoxP3DTR mice in different transplantation models. We used Cytof and FACS analysis for studying bone marrow (BM) populations and confocal microscopy and FACS-sorting for BM stromal cell and IL7 studies.

Results: The non-viral transduction protocol combined to the large scale production process minimally affected the phenotype of the CD19 CAR CIK-cell final product. Stable expression of CD19 CAR (average 65%) was achieved together with an efficient T cell expansion suitable for clinical application. Furthermore, modified cells displayed persistence of cell subsets with memory phenotype, specific and effective anti-tumor activity. Upon comparison with conventional T-SB platforms, our method achieved superior results in terms of expansion, CAR expression and functionality. Adoptive transfer of CD19.CAR lymphocytes led to a significant antitumor response *in vivo*. CD19.CAR CIK cells also controlled leukemia in xenograft models of human ALL, bearing the high-risk features of MLL–ENL and Ph-like (PAX5/AUTS2) gene rearrangements. Frozen/thawed CD19.CAR CIK cells remained active *in vitro* and *in vivo* with efficacy comparable with that of fresh CIK cells. Furthermore, NOD-SCID-γchain-/- (NSG) mice were treated with CD19.CAR CIK cells to evaluate general toxicity, tissue damage, and biodistribution. Notably, we found no evidence of integration enrichment near cancer-related genes and transposase expression in the final cell product.

Conclusions: Our findings indicate that Treg act as a key regulator of B cell differentiation promoting production of mature B cells through an ICAM1+CD31- perivascular stromal cells/IL7 mediated mechanism that is not dependent on alloantigen recognition, therefore Treg are critical in building the donor HSC and B cell precursor niche. Finally, Treg adoptive transfer induces immune reconstitution and tolerance to BM grafts providing a new tool for translation especially in children with SCID or hemoglobinopathies.

C052

BALANCE OF ANTI-CD123 CHIMERIC ANTIGEN RECEPTOR (CAR) BINDING AFFINITY AND DENSITY IN AN *in vitro* MODEL OF ACUTE MYELOID LEUKEMIA

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Chimeric Antigen Receptors (CARs)-redirected T lymphocytes are a promising novel immunotherapeutic approach, nowadays object of accurate preclinical evaluation also for the treatment of Acute Myeloid Leukemia (AML). In this context, we recently developed a CAR against CD123, over-expressed on AML blasts and leukemic stem cells. However, the potential recognition of low CD123-positive healthy tissues, through the "on-target-off-organ" effect, limits the safe clinical employment of CAR-redirected T cells. Therefore, in search for a CAR design optimization, we here evaluated the effect of variables capable to modulate CAR T-cell functional profiles in a context-dependent manner, such as CAR binding affinity for the target antigen, CAR expression and target antigen density. To study these variables in the absence of other interfering elements we exploited computational structural biology tools to design rational mutations in the anti-CD123 CAR antigen binding domain that altered CAR expression and CAR binding affinity, without affecting the overall CAR design. We were able to define both "lytic" and "activation" antigen thresholds, showing that whereas the early T-cell cytotoxic activity is not affected either by CAR expression or CAR affinity tuning, later effector functions are impaired by low CAR expression. Moreover, a promising balance in the efficacy and safety profiles of CAR T cells was observed in the lowest affinity mutant in response to targets with different antigen densities. Overall, the full dissection of all these variables offers additional knowledge for the proper design of a suitable anti-CD123 CAR for the treatment of AML.

C053

PRECLINICAL EVALUATION OF NON-VIRAL MODIFIED CAR+ LYMPHOCYTES FOR THE TREATMENT OF ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction: Chimeric antigen receptor (CAR)-modified T-cell adoptive immunotherapy has emerged as promising therapeutic option proven effective in the treatment of hematological malignancies. However, the success of CAR-engineered T cells strictly depends on the optimization of several critical parameters related to cell manufacturing and gene therapy. Therefore, we sought to develop a novel gene-modification protocol to engineer cytokine-induced killer cells (CIKs) with CD19 CAR using the Sleeping Beauty (SB) transposon system for the clinical application.

Methods: With an improved SB transposon platform, we genetically modified CIK cells to express the CAR specific for acute lymphoblastic leukemia (ALL) CD19+ blasts and evaluated their preclinical efficacy and safety.

Results: The non-viral transduction protocol combined to the large scale production process minimally affected the phenotype of the CD19 CAR CIK-cell final product. Stable expression of CD19 CAR (average 65%) was achieved together with an efficient T cell expansion suitable for clinical application. Furthermore, modified cells displayed persistence of cell subsets with memory phenotype, specific and effective anti-tumor activity. Upon comparison with conventional T-SB platforms, our method achieved superior results in terms of expansion, CAR expression and functionality. Adoptive transfer of CD19.CAR lymphocytes led to a significant antitumor response *in vivo*. CD19.CAR CIK cells also controlled leukemia in xenograft models of human ALL, bearing the high-risk features of MLL–ENL and Ph-like (PAX5/AUTS2) gene rearrangements. Frozen/thawed CD19.CAR CIK cells remained active in vitro and *in vivo* with efficacy comparable with that of fresh CIK cells. Furthermore, NOD-SCID-γchain-/- (NSG) mice were treated with CD19.CAR CIK cells to evaluate general toxicity, tissue damage, and biodistribution. Notably, we found no evidence of integration enrichment near cancer-related genes and trans-

posase expression in the final cell product.

Conclusions: Taken all together, our findings describe a novel donor-derived non-viral CAR approach characterised by efficient cell transfection and expansion that may widen the range of applications of T cell-based immunotherapy. We are currently designing a phase I/II study for relapsing and remitting ALL after Haematopoietic Stem Cell Transplantation (HSCT).

C054

BAFF RECEPTOR IS A POTENTIAL TARGET FOR A MORE SELECTIVE CHIMERIC ANTIGEN RECEPTOR (CAR)-MEDIATED TREATMENT OF B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL)

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Introduction: B-cell Acute Lymphoblastic Leukemia (B-ALL) is most common in children (80%) but it has also a peak of incidence in adult age. Recently, new immunotherapeutic approaches targeting the CD19 molecule paved the way for the treatment of relapsed and refractory lymphoblastic leukemia, which remains a major therapeutic challenge. Important downsides of these strategies are the emergence of CD19-negative relapses in 10% of treated patients and B-cell aplasia. We hypothesized that BAFF Receptor (BAFF-R), a transmembrane protein which plays a key role in B-cell maturation and survival *in vivo*, could be an interesting molecule to be targeted.

Methods: We characterized the expression of BAFF-R in B-ALL primary samples. As immunotherapeutic approach to target BAFF-R molecule, we developed six anti-BAFFR.CARs that differ for the inversion of the VH and VL and the length of the spacer domain.

Results: We found that BAFF-R is over-expressed on B-ALL leukemic blasts at the onset (44,36%, n=20) and it is slightly increased at relapse (54,91%, n=20). On the other hand, the expression of BAFF-R is absent or very low in normal tissues and on bone marrow B-cell precursors. Cytokine-induced Killer (CIK) cells, engineered using the Sleeping Beauty (SB) transposon system, stably expressed anti-BAFFR.CARs (average 60%) and maintained their characteristic phenotype. The newly constructed CARs demonstrated a specific anti-leukemic activity towards target cells, such as NALM-6, and, in particular, the shortest VHVL CAR exerted the higher efficacy compared to the other constructs with an *in vitro* killing activity of 60%. We also evaluated later effector functions in terms of cytokine release by intracellular staining (16% of IFN-Œ≥ and 35% of IL-2 producing cells). Importantly, we also detected a cytotoxic activity towards primary B-ALL blasts (average 63%, n=4) by the shortest VHVL CAR.

Conclusions: These findings make this receptor an attractive target for a second line B-ALL immunotherapy in case of relapse after CD19-targeting therapies, also suggesting that this strategy could limit B-cell aplasia.

C055

SPECIFIC TARGETING OF ACUTE MYELOID LEUKEMIA BY THE USE OF ENGINEERED CIK (CYTOKINE-INDUCED KILLER) CELLS EXPRESSING THE ANTI-CD33 CHIMERIC ANTIGEN RECEPTOR (CAR)

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Introduction: Acute Myeloid Leukemia (AML) is an aggressive malignancy still associated with high relapse rates when treated with conventional chemotherapeutic and hematopoietic transplantation regimens. In search for alternative strategies, immunotherapy adopting T cells redirected with Chimeric Antigen Receptors (CARs) represents an innovative route of investigation.

The CD33 myeloid antigen is broadly expressed on AML blasts, representing a suitable antigen to be targeted with CAR-T cells.

Methods: Here we proved the feasibility of harnessing Cytokine Induced killer (CIK) effector cells with a third generation anti-CD33 CAR through the non viral Sleeping-Beauty transposon system, starting from fresh and frozen healthy mononuclear cells (PBMCs) and also from frozen primary AML samples. The efficacy profile of anti-CD33 CAR-CIK cells against both AML cell lines and primary AML samples has been evaluated by means of *in vitro* assays such as cytotoxicity, cytokine production and proliferation.

Results: Anti-CD33 CAR-CIK cells were able to induce a potent anti-leukemic activity as compared to unmanipulated CIK cells, in terms of specific killing (up to 70%), proliferation (up to 40% of Ki67+CAR-CIK cells) and cytokine production (up to 30% for both IL-2 and IFN-gamma producing CAR-CIK cells) when challenged with both AML cell lines and primary leukemic cells.

Conclusions: The *in vitro* efficacy of anti-CD33 CAR-CIK cells against AML prompted us to develop an *in vivo* chemotherapy xenograft mouse model to better resemble the clinical human setting of relapsed/refractory AML. This model will be exploited to investigate the efficacy of the anti-CD33 CAR-CIK cells immunotherapy on the resistant/residual AML cells that were not eradicated by the standard chemotherapy. Moreover, envisaging a safer clinical translation of this immunotherapeutic approach, an mRNA anti-CD33 CAR strategy is currently under investigation, in order to limit the potential myelotoxicity due to the long-term off-target effect on normal hematopoietic stem/myeloid progenitor cells.

C056

ABCA1 MEDIATES THE EFFLUX OF EXTRACELLULAR ISOPENTENYL-PYROPHOSPHATE AND THE ACTIVATION OF Vγ9Vδ2 T-CELL

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Introduction: Phosphorylated metabolites of mevalonate (Mev) pathway like isopentenyl-pyrophosphate (IPP) induce proliferation of Vγ9Vδ2 T-cells. Endogenous IPP levels can be lowered or increased by aminobisphosphonates (NBPs) such as zoledronic acid (ZA). We have shown that soluble IPP released by dendritic cells (DC)ZA+ is a potent Vγ9Vδ2 T-cell activator and so we have verified the ability of a variety of tumoral and non-tumoral cells to activate Vγ9Vδ2 T-cells and to release extracellular IPP. The aim of this work was to identified the protein involved in IPP efflux.

Methods: γδ T cell proliferation: PBMC and PBL from CTRL were stimulated for 7 days with the supernatants obtained from autologous DCZA- and DCZA+ and 10 IU/mL IL-2 to evaluate their ability to induce Vγ9Vδ2 T cell proliferation. IPP efflux:cells were radiolabelled with 1 microCi/ml [3H]-acetate; after 24 h, cell supernatants were collected, [3H]-IPP was extracted and resolved by thin layer chromatography. After separation, the spot corresponding to IPP was cut and solubilized, and the radioactivity was quantified by liquid scintillation count. siRNA ABCA1: cells were incubated 96 h with a 20-25 nucleotide non targeting scrambled siRNA or specific ABCA1 siRNA (Accell Thermo Scientific), then lysed and subjected to the Western blot analysis of ABCA1 expression.

Results: We tested a variety of tumoral and non-tumoral cells for their ability to activate Vγ9Vδ2 T-cells and correlated this ability with the release of extracellular IPP. We used samples from healthy donors included peripheral blood (PB) monocytes, monocyte-derived DCs, and bone marrow (BM) stromal cells; samples from cancer patients included myeloma cells, chronic lymphocytic leukemia (CLL) cells, and BM stromal cells from multiple myeloma (MM) and CLL patients; tumor cell lines included the monocytic THP1 and histiocytic U937 cells and the myeloma cell line SKMMI. We found that the activation of Vγ9Vδ2 T-cells was proportional to the simultaneous efflux of IPP and to the activity/expression of ATP-binding cassette transporter A1 (ABCA1), a membrane transporter involved in the delivery of intracellular cholesterol to apoA-I.

We have demonstrated that the most potent IPP releaser cells had also the highest expression of ABCA1. Functional ABCA1 inhibition with probucol slightly increased intracellular IPP accumulation, but abrogated extracellular IPP release and Vγ9Vδ2 T-cell activation. Abca1 silencing experiments confirmed the key role played by ABCA1. ABCA1 is up-regulated by ZA via LXRα transcriptional activation induced by intracellular IPP accumulation and inhibition of the PI3K/Akt/mTOR signaling pathway.

Conclusions: These preliminary results suggest that ABCA1 is involved in extracellular IPP release and Vγ9Vδ2 T-cell activation induced by ZA-treated DC and therefore play a crucial role as mediator of the immune response.

Immunophenotyping and Minimal Residual Disease

C057

A TECHNICAL COMPARISON BETWEEN IGH AND BCL1/IGH MOLECULAR MARKERS FOR MINIMAL RESIDUAL DISEASE ANALYSIS IN MANTLE CELL LYMPHOMA

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Background: Minimal residual disease (MRD) is a strong outcome predictor in mantle cell lymphoma (MCL). Although the real time quantitative PCR approach is standardized in the context of the EuroMRD consortium, the highly sensitive qualitative nested PCR is still commonly used. Despite being a very sensitive method, it lacks standardization and validation on large patients series. Therefore, we compared the performance of nested PCR for the two MCL molecular markers: the immunoglobulin heavy chain gene rearrangement IGH, based on Allele Specific Oligonucleotide (ASO) PCR and the BCL1/IGH rearrangement, based on consensus primers PCR approach.

Patients and Methods: Paired bone marrow (BM) and peripheral blood (PB) samples, from MCL patients, receiving first or second-line immunochemotherapy were collected at: baseline, midterm chemo, end of induction and, only in younger patients, after consolidation with autologous transplantation (ASCT). Screening for IGH and BCL1/IGH molecular marker was done at baseline and MRD analysis was performed by nested PCR on both markers. Differences between markers negativization rates were calculated at each timepoint by chi square test.

	Midterm chemo		End of induction		Post ASCT	
	BM	PB	BM	PB	BM	PB
Total patients	63	64	51	52	43	42
IGH + BCL1 +	28 (44%)	21 (33%)	5 (10%)	2 (4%)	10 (23%)	5 (12%)
IGH + BCL1 –	13 (21%)	13 (20%)	19 (37%)	10 (19%)	17 (39%)	10 (24%)
IGH – BCL1 –	15 (24%)	24 (37%)	24 (47%)	30 (58%)	15 (35%)	26 (62%)
IGH – BCL1 +	4 (6%)	4 (6%)	1 (2%)	8 (15%)	1 (2%)	0
Non evaluable	3 (5%)	2 (3%)	2 (4%)	2 (4%)	0	1 (2%)
Tot concordant	43 (68%)	45 (70%)	29 (57%)	32 (61%)	25 (58%)	31 (74%)

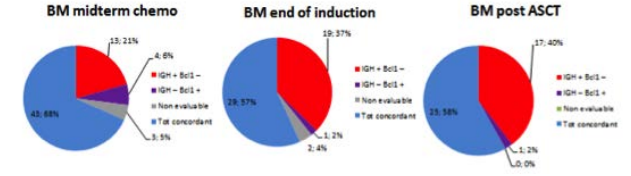

Figure 1 and Table 1.

Results: Between 2010 and 2015, BM and PB from 380 MCL patients were screened for a molecular marker: 272 received high dose chemoimmunotherapy followed by ASCT and 108 conventional chemoimmunotherapy. A molecular marker was identified in 319/380 patients (84%): 247 (65%) had a IGH marker, 133 (35%) a BCL1/IGH and 74 (19%) both markers. Therefore, for patients carrying both markers, the concordance between MRD results was tested at different time points, ranging between 57 and 74%; however, a considerable fraction of samples showed discordant results between markers, moreover discordances increase in last follow up samples: most of them scored IGH+ and BCL1/IGH- (median 27%,

range 19-39%), while the opposite was less frequent (median 5%, range 0-15%) (Figure 1). In detail, the MRD negativization rates on BM based on IGH and BCL1/IGH marker, respectively, were: 30% (19/63) vs 44% (28/63) at midterm (p=ns), 49% (25/51) vs 84% (43/51) at the end of induction (p=0.0001) and 37% (15/43) vs 74% (32/43) post ASCT (p=0.0002). Similar results were observed on PB (Table 1).

Conclusions: Both IGH and BCL1/IGH molecular markers are currently used to monitor MRD in MCL patients. However, MRD analysis performed by qualitative nested PCR approach might lead to not superimposable results between the two targeted markers. Our findings, on patients carrying both markers, indicate an earlier negativization of BCL1/IGH compared to IGH. These results might indicate a higher reliability of the IGH marker in MRD monitoring, possibly due to the use of of different PCR approaches (ASO-PCR vs consensus-PCR). Correlation with clinical data is necessary to investigate the higher reliablity of IGH base MRD-PCR in predicting outcome.

C058

WT1 AND MULTIPARAMETER FLOW CYTOMETRY MRD STATUS AFTER CONSOLIDATION SIGNIFICANTLY PREDICTS EARLY RELAPSE IN A COHORT OF 266 ACUTE MYELOID LEUKEMIA PATIENTS

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Post induction and consolidation MRD might be an indipendent predictor of AML outcome. We have evaluated post induction and consolidation bone marrow mimimal residual disease (MRD) in 266 AML patients (median age: 59 years, range: 17-89) with 13 months median follow-up (range 1-107). We analysed abnormal leukemia immunophenotype (ALIP) by multiparameter flow cytometry (MPFC) and WT1 by RT-PCR as described by Buccisano et al and Cilloni et al. The molecular cytogenetic risk was available in 240 patients. We analysed overall and 1 yr Cumulative Incidence of Relapse (CIR) by MRD status, patients and disease characteristics, kind of post-consolidation treatment.

WT1 was +ve in 219/256 patients (85.5%) at diagnosis (median 2,320; range:0-268,784), in 71/201 (35%) post induction (median 78; range:0.4-134,633) and in 28/171 (16.4%) post consolidation (median 37; range:0.4-45,358). MPFC MRD was +ve in 48/160 (30%) patients after induction and in 52/127 (41%) after consolidation. 178/242 patients achieved CR after induction and 15 patients responded to salvage treatment accounting for 193 responder (79.8%), 82 relapsed in a median of 8,5 months (1-52 months) with 52.3% 5 yr CIR, and 29.7% 1 yr CIR (N: 50). Patients receiving chemotherapy (88), Autologous (27) and Allogeneic Transplant (103) as post-consolidation treatment had respectively 45%, 24% and 41% 1 yr CIR (p=0.01). ELN favorable patients had 25.6% 1 yr CIR, compared with 11.7% in NPM-FLT3 ITD-, 36.4% in FLT3 ITD+ patients and 52% in unfavorable ELN group (p=0.003). Patients aged more than 60 yrs had 50.6% 1 yr CIR compared with 24% observed in the younger counterpart (p=0.001).

We identified after induction a very poor setting of patients with double positive WT1 and MPFC MRD (double positive MRD) with 54.6% 1 yr CIR; an intermediate group with discordant WT1 and MPFC MRD results (discordant MRD) with 26.3% 1 yr CIR; a very good setting with negative WT1 and MPFC values (double negative) with 11% 1 yr CIR (p<0.0001).

Post-consolidation MRD analysis showed a 85% 1 yr CIR in patients with double positive MRD vs 29.5% in discordant MRD and 12.6% in double negative patients respectively (p<0.0001). Multivariate analysis confirmed the predictive role of post-consolidation MRD in terms of 1 yr CIR with 2.55 relative risk (RR) in discordant positive MRD and 20.28 RR in double positive MRD in comparison with double negative patients (p<0.0001). The analysis also confirmed the predictive role of age with a 2.67 RR of relapse at 1 yr in patients older than 59 yrs in comparison to younger patients (p=0.01). Patients with double positive, discordant and double negative post-consolidation MRD had respectively 73.3%, 25% and 4.5% 1 yr CIR post Allogeneic Transplant. Post-consolidation MRD is a reliable predictor of early relapse also in allogeneic setting, identifying an extremely poor setting of patients who should be enrolled in experimental protocol including MRD driven retreatment or maintenance.

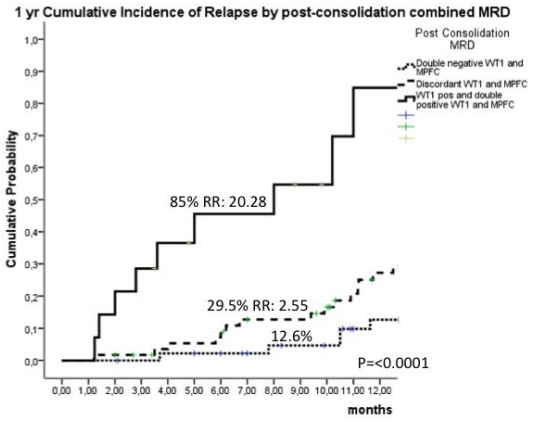

Figure 1.

C059

THE DIGITAL DROPLET PCR: A PROMISING MOLECULAR TOOL FOR MINIMAL RESIDUAL DISEASE DETECTION IN HAIRY CELL LEUKEMIA

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Introduction: The hairy cell leukemia (HCL) is a rare B-cell chronic lymphoproliferative disorder whose pathogenesis has been recently associated to the B-RAF V600E mutation. The digital droplet PCR (DD-PCR) is a new molecular technique that, without any standard reference curve and high sensitivity, is able to accurately quantitate DNA mutations; thus, it would be probably useful for detecting B-RAF mutation in HCL also.

Methods: Our study involved 47 patients with indolent non-Hodgkin's lymphoma, including 28 with HCL classic form, one with the variant form, and 18 with splenic marginal zone lymphoma (SMZL). The assessment of the BRAF V600E mutation was performed on bone marrow samples by both the Real Time PCR (QT-PCR) and the Droplet Digital PCR (DD-PCR); in the same samples the B clonality had been assessed by conventional qualitative fluorescent PCR for the IgH rearrangement. The aim of the study was the comparison of these three different molecular techniques in term of sensitivity and specificity and their applicability in the clinical setting of minimal residual disease (MRD) detection in HCL.

Results: The sensitivity of DD-PCR resulted about half a logarithm superior to that of QT-PCR and qualitative PCR ($1x10^{-3}$ vs $5x10^{-3}$). All patients were evaluable by the 3 techniques; all 18 patients with SMZL showed a B-cell clonality, but were B-RAF wild-type by both QT-PCR and DD-PCR. On the other hand, all HCL cases at diagnosis showed the IgH rearrangement and B-RAF mutation, either by QT-PCR or DD-PCR. The patient with the variant form showed the B-cell clonality but resulted B-RAF wild-type. After treatment with Rituximab and 2-Cloro-deoxi-adenosine, 27 cases were evaluable: 11 patients achieved a clinical partial response (PR) and 16 a complete remission (CR). After the molecular analysis, 3 of patients in CR still showed the IgH clonality and the B-RAF mutation by QT-PCR. Interestingly, 5 patients were still MRD-positive by DD-PCR. In the group of 11 patients in PR, 10 still presented IgH rearrangement; 9 presented the B-RAF mutation by QT-PCR and 10 by DD-PCR. Interestingly, one patient MRD-negative by QT-PCR were positive non quantifiable by DD-PCR. During follow-up, 7 patients maintained the CR and one relapsed; the relapsed one showed both the B-cell clonality and B-RAF mutation detected by both techniques. Of the cases in CR, 3/7 still showed the IgH clonality and 4/7 resulted still B-RAF mutated, by both techniques.

Conclusions: Our study shows: 1) the higher sensitivity of DD-PCR vs QT-PCR and qualitative PCR; 2) the specificity of the DD-PCR that is comparable to that of QT-PCR (no patient with marginal splenic lymphoma or HCL variant resulted B-RAF mutated). These results, in addition to the fact that DD-PCR is a quite simple technique with costs comparable to those of QT-PCR, support the introduction of DD-PCR in the scenario of the molecular techniques useful for diagnosis and follow-up of HCL.

C060

EFFICACY OF THE R-BENDAMUSTINE ON THE ERADICATION OF THE MOLECULAR MINIMAL RESIDUAL DISEASE IN FOLLICULAR LYMPHOMA

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Introduction: R-Bendamustine as treatment for follicular lymphoma (FL) is considered as a valid alternative to the R-CHOP. The FIL FOLL05 trial demonstrated the prognostic role of molecular minimal residual disease (MRD) and the ability of the R-CHOP of clearing MRD in a deeper way than R-CVP and R-FM. The FOLL12 study will give us further information about the "molecular power" of the R-bendamustine. In the meantime, we retrospectively evaluated the impact of R-Bendamustine on MRD in a multicentric series of FL cases.

Methods: Qualitative and quantitative PCR for the BCL2/JH rearrangement were performed as previously reported [Galimberti S, Clinical Cancer Res 2014]. Forty-five patients were enrolled on the basis of the availability of BM samples before and just after treatment.

Results: Twenty-three patients were male and twenty-two female; the median age was 63 years (range 36-83); 37 received R-bendamustine as first line, and 8 cases at the first relapse. Forty-one cases were in stage IV; 19% resulted at low, 65% at intermediate, and 16% at high FLIPI2 risk score. The BCL2/JH rearrangement was found in 29 cases by qualitative PCR (64.4%) and in 24 cases (53%) by quantitative PCR (two cases were positive for the mcr breakpoint and then not evaluable by quantitative PCR). Eighteen presented bone marrow infiltration; in 4 of them (22%) BCL2/JH rearrangement was negative, probably for the presence of a rearrangement involving other rare breakpoints. On the other hand, in 8 cases (44%) without BM infiltration at the microscopy, the molecular marker was found, possibly due to a sub-microscopic BM involvement. The quantization of the molecular tumor burden showed a great variability: the median value before treatment was 1.6x10-2 copies, ranging from 3x10-5 to 4x104. The BCL2/JH copy number did not correlate with stage and performance status, age, or gender, but was higher in patients presenting with high FLIPI2 score (p=0.07). During treatment, one patient died for infection; all remaining cases responded, with 4.5% of partial and 95.5% of complete responses. The presence of the molecular marker before treatment did not significantly impact on the quality of response, probably because of the very high overall response rate achieved. After treatment, 90% of cases became MRD-negative (either by qualitative or quantitative PCR); two cases resulted "positive but not quantifiable". The median reduction of the molecular tumor burden was about 4 logs, in respect of 2 logs already reported after R-CHOP and 2.5 reported after Yttrium-ibritumomab-tiuxetan [Ibatici A, Br J Haematology 2014]. With a median follow-up of 33 months, two patients relapsed; because of this very low number of events, it is not possible to evaluate the impact of the R-Bendamustine on OS and PFS.

Conclusions: This study, even if retrospective, clearly sustains the efficacy of the R-Bendamustine in the treatment of FL, both in term of clinical and molecular responses.

C061

IMPACT OF RESIDUAL DISEASE BEFORE ALLOGENEIC STEM CELL TRANSPLANTATION IN ADULT PATIENTS WITH PHILADELPHIA CHROMOSOME POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction: Allogeneic stem cell transplantation (alloHSCT) in first complete remission (CR1) remains the consolidation therapy of choice in Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL). Evidence is emerging that post-transplant relapse is influenced by the persistence of minimal residual disease (MRD), with an inferior outcome of patients undergoing transplantation with measurable level of MRD. Since a deeper molecular response can be achieved with innovative targeted therapies, such as second and third-generation Tyrosine Kinase Inhibitors (TKIs) or immunotherapy, an accurate evaluation of MRD values before alloHSCT may be very relevant. The aim of this study was to evaluate the impact of MRD levels before transplant in Ph+ ALL patients in CR1 on relapse incidence (RI), disease free survival

(DFS) and overall survival (OS).

Patients and methods: One hundred and six adult patients with newly diagnosed Ph+ ALL were enrolled into 2 prospective NILG protocols (09/00 ClinicalTrial.gov Identifier: NCT00358072 and 10/07 ClinicalTrial.gov Identifier: NCT00358072) and were treated with chemotherapy and Imatinib. One hundred (94%) achieved CR1 and 73 patients underwent an alloHSCT in CR1. Among these 73 patients, the MRD status measured at time of conditioning was available for 65 patients who are the subject of this report. MRD was determined by quantitative polymerase chain reaction (RQ-PCR) according to validated methods.

Results: A complete or major molecular response at time of conditioning was achieved in 24 patients (37%) (MRD- group), while 41 (63%) remained carriers of any other positive MRD level in the bone marrow or peripheral blood (MRD+ group). Patients' characteristics were similar between MRD+ and MRD- groups, except for a higher hemoglobin level and a predominance of male gender in MRD- group. The MRD negativity at time of conditioning was associated with a significant benefit in terms of risk of relapse at 5 years with a RI of 8% compared to 39% of patients with MRD positivity (p=0.007) (Figure 1). However, thanks to the post-transplant use of TKIs, the DFS probability was 58% vs 41% (p=0.17) and the OS was 58% vs 49% (p=0.55) in MRD negative compared to MRD positive patients, respectively (Figure 1). The cumulative incidence of non-relapse mortality was similar in the 2 groups.

Conclusions: Achieving a complete molecular remission before transplant reduce the risk of leukemia relapse even though TKIs may still rescue some patients relapsing after transplant. Accordingly, an effort to achieve a convincing molecular CR should be pursued and considered an essential prerequisite for successful alloHSCT.

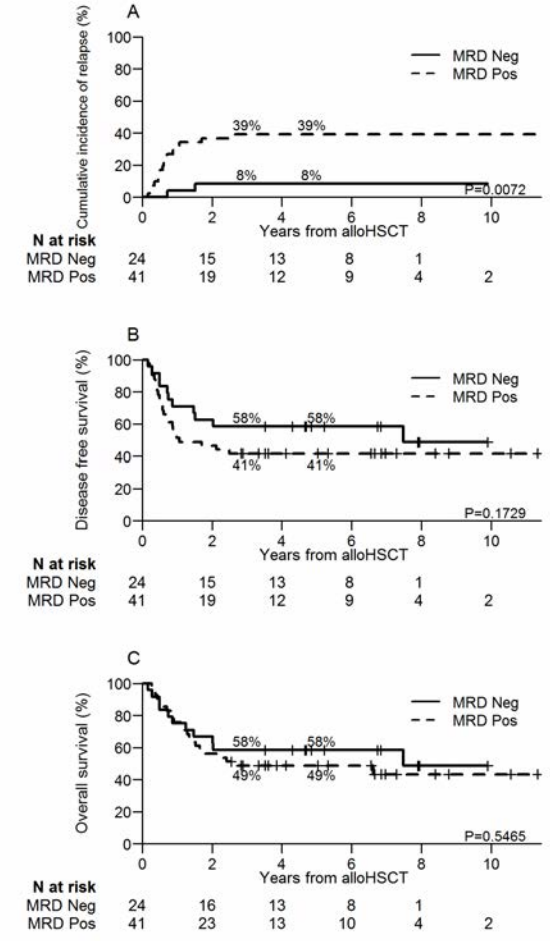

Figure 1.

C062

QUANTIFICATION OF THE JAK2V617F ALLELE BURDEN BY DROPLET DIGITAL PCR (DDPCR): APPLICATION TO MINIMAL RESIDUAL DISEASE (MRD) EVALUATION AFTER ALLOGENEIC STEM CELL TRANSPLANTATION IN PATIENTS AFFECTED BY MYELOPROLIFERATIVE NEOPLASMS (MPN)

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Introduction: Allogeneic Stem Cell Transplantation (ASCT) is the only curative treatment option for patients with Myelofibrosis. The most frequent phenotype-driving mutation in Myeloproliferative Neoplasms (MPN) patients is the V617F mutation in the JAK2 gene. A high sensitive quantification of JAK2V617F mutation load can be useful to assess Minimal Residual Disease (MRD) in treatment directed to eradicate the malignant clone, such as ASCT. Droplet Digital PCR (ddPCR) is a quantitative approach for the detection of rare allele characterized by a high level of sensitivity and specificity. We evaluated the efficacy of ddPCR JAK2V617F mutation detection assay in monitoring the MRD level in MPN patients who underwent to ASCT.

Methods: DNA from 9 patients affected by primary or secondary Myelofibrosis or by a secondary Acute Myeloid Leukemia derived from a previous MPN were collected serially during the follow-up after ASCT (50-2500 days). These samples were investigated for hematologic chimerism by PowerPlex System (Promega, USA) and were also analyzed for JAK2V617F mutation both by conventional allele specific PCR (ASO-PCR) and by a validated ddPCR mutation detection assay (Bio-rad, USA). Results were expressed as percentage of JAK2V617F mutated alleles on total alleles evaluated.

Results: The JAK2V617F ddPCR mutation assay was able to detect low mutation load (up to 0.006%), demonstrating to be much more sensitive than ASO-PCR (1-5%). In 4 patients, early after transplantation, we observed by ddPCR a low level of MRD that progressively increased during the follow-up and anticipated a decreased level of donor chimerism and a worsening clinical situation. In 4 other patients, who always showed a full donor chimerism and complete hematologic remission of the disease, very low levels of MRD (ranging from 1% to 0.006%) could be detected by ddPCR in the 2 years after ASCT. With a longer follow-up, a full molecular remission was achieved as demonstrated by ddPCR. Interestingly, in one patient whose post-transplant hematopoiesis proved full donor and negative for JAK2V617F mutation for 2 years, a late positive signal by ddPCR (0.075%) became apparent and heralded an extra-hematologic relapse (skin and bone). A subsequent second allogeneic transplant from the same sibling donor restored clinical and molecular remission.

Conclusions: The ddPCR proved to be a sensitive and accurate method in detecting JAK2V617F mutation. Therefore, this assay can be a valid tool for the MRD monitoring in JAK2V617F MPN patients undergone to ASCT. However, the use of this highly sensitive PCR should be considered with caution in the clinical management of transplanted patients to avoid inappropriate use of donor leukocyte infusion (DLI) and tampering of immunosuppression. A large number of patients have to be studied with ddPCR to better understand the clinical significance of low mutation load.

C063

NEXT-GENERATION-SEQUENCING BY 454 GS TECHNOLOGIES FOR MINIMAL RESIDUAL DETECTION IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. Minimal residual disease (MRD) detection has become routine clinical practice in many protocols of acute lymphoblastic leukemia (ALL) treatment. The gold standard approach for ALL-MRD monitoring is the use of allele-specific oligonucleotide quantitative real-time PCR (ASO-RQ-PCR) analysis of the junctional regions (VDJ) of rearranged immunoglobulin (IGH) and T-cell receptor (TCR) genes. Nevertheless the ASO-RQ-PCR MRD method requires extensive knowledge and is laborious and time-consuming. Recently, some studies assessed a next-generation sequencing (NGS) approach with a high throughput sequencing of VDJ rearrangements to evaluate MRD, this technique could combine the benefits of high sensitivity with none patients customization. To assess whether NGS technique is sensitive and specific, we compared, in B-ALL, IGH/TCR gene rearrangements screening and MRD detection by standard ASO-RQ-PCR with a new NGS approach.

Methods. We performed IGH/TCR screening according to BIOMED-2 protocol, ASO-RQ-PCR to set MRD was performed according to EURO-MRD-ALL guidelines. To assess NGS method we used for IGH/TCR screening and MRD detection the same BIOMED-2 primers adapted to 454 GS Junior Roche. An advantage of 454 over other NGS platform is the ability to produce long sequence reads; BIOMED-2 consensus primers give rise to amplicons of 340 bp that is the standard length of 454 pyrosequencing.

Table 1.

	Sensivity	QR	MRD TP1	MRD TP2	MRD TP3
VH6-1*01 EURO-MRD	1x10E-5	1x10E-4	1x10E-3		
VH6-1*01 NGS-MRD	1x10E-4	1x10E-4	0.9x10E-4		
VH3-30-3*01 EURO-MRD	1x10E-5	1x10E-4	5x10E-4	2x10E-4	NEG
VH3-30-3*01 NGS-MRD	5x10E-5	5x10E-5	1x10E-4	1x10E-4	NEG
TRG V8*01 EURO-MRD	1x10E-5	1x10E-4	5x10E-4	2x10E-4	NEG
TRG V8*01 NGS-MRD	5x10E-5	5x10E-5	3x10E-4	3x10E-4	NEG

Results and Conclusion. We identified on 3 ALL sample, through IgH/TCR screening rearrangements based on PCR-BIOMED-2, leukemia-derived sequences. NGS IGH/TCR screening was performed analyzing the obtained 454 run sequences to a cluster analysis by CD-HIT software. To define gene rearrangements associated to leukemia we used a frequency threshold of 5%. We tested 3 clonotypes, (VH6-1*01; VH3-30-3*01; TRGV8*01) for MRD detection by NGS approach at different time points. For MRD detection we analyzed the 454 run sequences by the application CD-HIT-EST-2D, this tool compares 2 datasets above a threshold of homology. The leukemia-derived sequence was used as target to compare (threshold of 0.99) the sequences obtained from NGS analysis of MRD sample. To determine the absolute measure of the total leukemia-derived molecules present in the follow-up sample (MRD sample) we added a known quantity of reference IGH/TCR sequence into the reaction and counted the associated sequencing reads (control spike-in). Finally we compared NGS-MRD results with ASO-RQ-PCR-MRD results. NGS-MRD evidenced high specificity with sensitivity between 10E-4 and 10E-5 and MRD evaluated on 7 follow-up points show comparable MRD value detected by ASO-RQ-PCR. (Table 1). In this report we described our experience related to the ALL-MRD detection by the new NGS method. This innovative approach looks promising because it is efficient and less time consuming. Further effort will be made to answer the need of standardization of NGS-MRD approach to be applicable in different laboratories.

C064

DNMT3A MUTATIONS ARE NOT A RELIABLE MARKER OF MRD IN AML

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Mutations in the DNMT3A gene have been reported in ~20% of patients with AML, with most common mutations affecting the R882 position. While initial reports suggested a negative impact of DNMT3A R882H on AML outcome, more recent studies have questioned its prognostic role. In this study, we aimed to clarify the prognostic impact of DNMT3A mutations in AML by analysing the kinetics of MRD and to identify the prognostic value of additional mutations. We analysed bone marrow samples of 556 de novo AML patients (median age=49 years, range 16-89) enrolled in GIMEMA multicentre trials and treated with standard chemotherapy. The molecular status of NPM1, FLT3 and DNMT3A R882 were analysed using standard methods. The expression of DNMT3A R882H, FLT3-ITD and NPM1-typeA mutations were also investigated in follow-up samples. The analysis on FACS-sorted cells was performed using specific markers for LAIP subpopulations. Longitudinal RQ-PCR assessment of DNMT3A R882 transcripts was carried out using a technique established by our group.

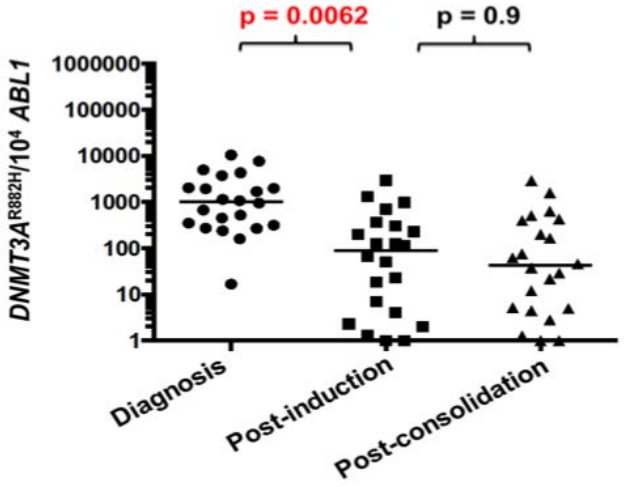

Figure 1. Kinetic evaluation of the DNMT3A R882H expression in 22 AML patients after induction and consolidation therapy.

Finally, additional mutations were analysed using Ion AmpliSeq™ AML panel on the Ion PGM™ platform (Life-Technologies) and Sanger sequencing. Fifty-seven of 556 AML patients (10%) were DNMT3A R882-mutated (42 R882H, 13 R882C and 2 R882S). We then concentrated on a subset of 22 DNMT3A R882H-mutated patients, with available samples collected at diag-

nosis, post-induction and post-consolidation therapy. Thirteen of them also presented a FLT3-mutation, while 17 where type A NPM1-mutated. NGS and Sanger sequencing for recurrent mutations in AML did not identify additional mutations associated with any of these markers. Looking at DNMT3A R882H kinetics by RQ-PCR, we found that DNMT3A R882H expression significantly decreased after induction treatment (Figure 1) in particular in FLT3 mutated patients as compared to patients with wild-type FLT3. However, DNMT3A levels remained stable after consolidation treatment. In addition, during follow-up, no significant differences were observed in the kinetics of DNMT3A levels in patients who relapsed as compared to those in continuous complete remission. Conversely, were able to confirm the prognostic value of longitudinal NPM1 mutation monitoring. In fact, NPM1-mutated transcripts significantly decreased after induction treatment and increased in 10 patients to levels similar to that of primary diagnostic sample at the time of relapse. In 2 patients, we sorted CD34+/CD33+/CD117± myeloid precursors, monocytes and lymphocytes. DNMT3A levels were high in myeloid progenitors and monocytes, and low in lymphocytes. A BM sample was available for one of the patients at the time of relapse at 12 months from diagnosis. All sub-populations were FLT3-ITD positive/NPM1-mutated and expressed very low levels of DNMT3A at the time of relapse, indicating emergence of a DNMT3A-negative clone. Our findings indicate that DNMT3A R882H expression is not a reliable marker for MRD, while they confirm the high prognostic value of longitudinal monitoring of NPM1 mutations. Our data also suggest that the kinetics of DNMT3A may follow the disease course in FLT3-mutated patients only.

Monoclonal Gammopathies and Multiple Myeloma 2

C065

THE ROLE OF RHOU GTPASE IN THE CROSS-TALK BETWEEN NEOPLASTIC PLASMA CELLS AND BONE MARROW MICROENVIRONMENT IN MULTIPLE MYELOMA

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Introduction: Multiple myeloma (MM), a plasma cell malignancy accounting for about 13% of all blood tumors, remains until today incurable. Malignant plasma cells that resist chemotherapy and repopulate the bone marrow (BM) are responsible for patients' relapse. Over the last few years a lot of attention has been drawn to the BM microenvironment and to the interaction between MM cells and bone marrow stromal cells (BMSCs). BMSC-produced soluble factors, like the IL-6 cytokine, are believed to impinge on MM intracellular signaling and cytoskeletal properties, protecting it from cytotoxic agents. Rho GTPases, in their active GTP-bound state, interact with effector proteins to control cytoskeleton remodeling, cell adhesion/polarization, and other essential processes for cell-cell interaction. The atypical Rho protein RhoU displays spontaneous activation and is expressed at low levels in most tissues and organs. This protein might mediate the effects of IL6R/STAT3 signaling in inducing filopodium formation and stress fiber dissolution, both critical steps in promoting cell motility. While typical Rho proteins (that share significant sequence homology with RhoU) as Cdc42 and Rac-1 have an established role in cancer, very little is known on RhoU in tumorigenesis, in particular in hematologic malignancies.

Methods: Since the IL6R/STAT3 signaling is of great importance in MM malignancy, we have endeavored to study RhoU expression by qRT-PCR in normal versus MM plasma cells. We also focused on understanding its localization by immunofluorescence in these cells. Lastly, we aimed at unraveling the mechanisms through which RhoU expression is regulated in MM cells in the context of BM microenvironment.

Results: Here we provide data showing that RhoU GTPase is widely overexpressed in malignant plasma cells from BM samples of MM patients and in IL-6 dependent MM cell lines, but not in malignant plasma cells purified from the peripheral blood of Plasma Cell Leukemia (PCL) patients. We also demonstrate that its expression is positively modulated through the activation of STAT3 transcription factor by IL-6, a major growth factor for MM cells, and to the same extent by BMSC derived soluble factors. A blockade in STAT3 activation, by using its commercially available inhibitor STATTIC, led to a marked decrease in RhoU mRNA transcription in a dose dependent manner, and caused a clear impairment in migration/motility capacity of these cells We describe for the first time a localization of this G protein in the nucleolus of BMSC/IL-6 dependent MM cells. Preliminary results on the effects of Lenalidomide, known to disrupt microenvironment signaling, on RhoU expression will also be discussed.

Conclusions: These results put to light the pleiotropic features that RhoU could display in this malignancy. We believe that RhoU might have an important role in MM pathogenesis and could become a suitable target to disrupt the MM plasma cell interaction with protective BM niches.

C066

CLONAL EVOLUTION IN PLASMA CELL DISORDERS: CHANGES OF CHROMOSOMAL ABNORMALITIES WITH A PROGNOSTIC SIGNIFICANCE DURING DISEASE PROGRESSION

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Introduction: Plasma cell (PC) disorders are characterized by the presence of several chromosomal abnormalities and clonal evolution during disease progression. In the present study the most common genetic anomalies with different prognostic values in bone marrow (BM) samples from Monoclonal Gammopathy of Undetermined Significance (MGUS), Smouldering Myeloma (SM), Multiple Myeloma (MM) and Plasma Cell Leukemia (PCL) patients have been compared at different timepoints from diagnosis to progression/relapse. Flow-cytometry represents the main tool for PC neoplasms characterization and monitoring. We also investigated if BMPC immunophenotypic parameters changed together with chromosomal abnormalities.

Methods: Between 2002 and 2016, BM samples from 117 patients affected by PC neoplasms were collected in our Hematology division at several timepoints for immunophenotypic and FISH analyses. At the first timepoint, 4 patients were at the stage of MGUS, 15 SM, 2 PCL and 96 MM at diagnosis. Subsequent timepoints included disease progression or relapse. FISH was performed on purified BMPC using anti-CD138-coated magnetic beads. Nuclei from fixed PC were prepared for interphase FISH using standard methods. DNA probes were used to detect Rb1 and TP53 deletions, t(11;14)(q13;q32), t(4;14)(p16;q32) and t(14;16)(q32;q23). PC immunophenotype was assessed using four to six-colours combinations of the following MoAbs: CD38, CD138, CD56, CD45, CD19, CD20, CD117, cytoplasmic kappa/lambda.

Results: Almost one third of all the patients (36/117, 30.8%) showed changes in terms of FISH results during disease progression. In 29/36 patients (80.6%), gain of new chromosomal abnormalities was observed, mainly Rb1 and TP53 deletions, suggesting a linear pathway of clonal evolution and confirming that IGH translocations are, indeed, primary genetic events. In 7/36 patients (19.4%) loss of previously detected genetic anomalies was observed and in 2 of them gain of new ones was detected, suggesting the selection of a novel dominant neoplastic clone, probably following a branching pathway. Our results confirm that genetic instability increases as disease progresses. In fact, the value of 30.8%, obtained considering patients relapsed after two or more lines of therapy, decreased to 21.1% evaluating MGUS and SM patients only, during disease progression to MM and 20.4% considering changes from MM at diagnosis to first relapse. Moreover, by comparing early first relapse (<=24 months) with late first relapse (>24 months) no significant difference was highlighted in terms of genetic changing rate (21.1% vs 23.3%, p=0.833). The evaluation of immunophenotype showed some changes during disease progression but there was no statistically significant difference between patients with or without clonal evolution detected by FISH analyses.

Conclusions: These results will be compared with clinical data in order to understand the impact of therapy on clonal evolution in PC disorders.

C067

CLINICAL CHARACTERISTICS AND TREATMENT PATTERNS AMONG ITALIAN PATIENTS WITH RELAPSED REFRACTORY MULTIPLE MYELOMA: RESULTS FROM A PROSPECTIVE OBSERVATIONAL STUDY, PREAMBLE

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Background: Knowledge of clinical practice and patient characteristics in relapsed refractory multiple myeloma (RRMM) can inform and improve national treatment strategies.

Aims: To describe the clinical and treatment characteristics of patients (pts) with RRMM in Italy.

Table. Characteristics of the Italian treated population in the PREAMBLE study

n (%) unless otherwise stated	All Italian patients (N=197)	IMiD-based regimens [a] (N=101)	PI-based regimens [b] (N=92)	IMiD+PI-based regimens (N=4)
Demographics				
Sex				
Male	113 (57.4)	57 (56..4)	53 (57.6)	3 (75.0)
Female	84 (42.6)	39 (42.4)	39 (42.4)	1 (25.0)
Age				
mean years (SD)	68.0 (8.8)	67.1 (9.2)	68.8 (9.7)	68.8 (9.74)
<65 years	65 (33.0)	31 (30.7)	33 (35.9)	1 (25.0)
65-75 years	83 (42.1)	44 (43.6)	37 (40.2)	2 (50.0)
>75 years	49 (24.9)	26 (25.7)	22 (23.9)	1 (25.0)
Disease and treatment history				
Number of lines of prior MM therapy				
1	103 (52.3)	56 (55.4)	44 (47.8)	3 (75.0)
2	54 (27.4)	31 (30.7)	23 (25.0)	0 (0.0)
3	24 (12.2)	10 (9.9)	14 (15.2)	0 (0.0)
>3	16 (8.1)	4 (4.0)	11 (12.0)	1 (25.0)
ISS stage at study entry				
Known	139 (70.6)	69 (68.3)	67 (72.8)	3 (75.0)
I [c]	61 (43.9)	28 (40.6)	31 (46.3)	2 (66.7)
II [c]	46 (33.1)	23 (33.3)	22 (32.8)	1 (33.3)
III [c]	32 (23.0)	18 (26.1)	14 (20.9)	0 (0.0)
Unknown	56 (28.4)	30 (29.7)	25 (27.2)	1 (25.0)
Not reported	2 (1.0)	2 (2.0)	0	0
Prior transplantation	86 (43.7)	37 (36.6)	46 (50.0)	3 (75.0)
Risk category at study entry				
Evaluated	15 (7.6)	10 (9.9)	5 (5.4)	0
Low risk [d]	0	0	0	0
Standard risk [d]	12 (80.0)	9 (90.0)	3 (60.0)	0
High risk [d]	3 (20.0)	1 (10.0)	2 (40.0)	0
Not evaluated	182 (92.4)	91 (90.1)	87 (94.6)	4 (100)
Chromosomal abnormalities				
Reported	18 (9.1)	11 (10.9)	7 (7.6)	0
Yes [e]	12 (66.7)	7 (63.6)	5 (71.4)	0
No [e]	6 (33.3)	4 (36.4)	2 (28.6)	0
Not reported	179 (90.9)	90 (89.1)	85 (92.4)	4 (100)
Other medical history				
Number of comorbidities				
0	55 (27.9)	19 (18.8)	36 (39.1)	0
1	50 (25.4)	28 (27.7)	21 (22.8)	1 (25.0)
2	41 (20.8)	22 (21.8)	18 (19.6)	1 (25.0)
3	20 (10.2)	14 (13.9)	6 (6.5)	0
4	18 (9.1)	9 (8.9)	8 (8.7)	1 (25.0)
5	7 (3.6)	5 (5.0)	1 (1.1)	1 (25.0)
>5	6 (3.0)	4 (4.0)	2 (2.2)	0
Type of comorbidity				
Cardiovascular disorders	78 (39.6)	45 (44.6)	30 (32.6)	3 (75.0)
Metabolism and nutrition disorders	32 (16.2)	19 (18.8)	12 (13.0)	1 (25.0)
Musculoskeletal and connective tissue disorders	17 (8.6)	7 (6.9)	9 (9.8)	1 (25.0)
Renal and urinary disorders	17 (8.6)	9 (8.9)	7 (7.6)	1 (25.0)
Nervous system disorders	13 (6.6)	8 (7.9)	5 (5.4)	0

[a] Including lenalidomide, thalidomide and pomalidomide; [b] Including bortezomib and carfilzomib; [c] Percentages based on number of patients with known ISS stage; [d] Percentages based on number of patients with evaluated risk category; [e] Percentages based on number of patients where data are reported

Methods: PREAMBLE (NCT01838512) is a prospective, multimarket (US, Canada, Italy, France, UK, Germany), observational study, with up to 3 years of follow up per pt. Eligible pts have ≥1 prior therapy for MM with disease progression from the most recent therapy, and have initiated index therapy (with one or a combination of immunomodulatory drugs (IMiDs), proteasome inhibitors (PIs), monoclonal antibodies, histone deacetylase inhibitors, Akt inhibitors, novel combination therapies, corticosteroids, or cytotoxic regimens) within 90 days prior to, or 30 days after, study consent. Demographics, treatment and medical history data were collected, including diagnostic tests, MM treatments and concomitant medications. Descriptive statistics relating to pts in Italy from an interim analysis are reported.

Results: At the cut-off date (December 7th 2015), 197 pts had enrolled and received treatment in Italy, with a median follow-up of 15.1 (Q1–Q3: 8–24) months. Pts were mostly male (57%) with

mean age 68 years (Table), and began index therapy after a mean of 51.8 months since initial diagnosis. At study entry, more pts were relapsed (76.5%) than refractory (24%), and most (79%) were managed in a university hospital. The majority of pts were ISS stage I (44 %) or II (33%), though many were of an unknown stage (28%). Seventy-two percent of pts had comorbidities, commonly one (25%) or two (21%), though some pts (3%) had over five. Cardiovascular (40%) and metabolic/nutrition (16%) disorders were most frequent, while musculoskeletal/connective tissue (9%), renal/urinary (9%) and nervous system disorders (7%) were less common. 39% of pts receiving PIs and 19% of pts receiving IMiDs had no comorbidities; one comorbidity was, however, most common (28%) of pts receiving IMiDs. In the Italian cohort, 101 pts (51%) received IMiDs, 92 (47%) PIs and 4 (2%) IMiDs+PIs as their index RRMM therapy. The most common agent prescribed amongst IMiDs was lenalidomide (98.9%) and amongst PIs, bortezomib (100%), both in combination with dexamethasone. Duration of index therapy varied between treatment types: median 8.8 (Q1–Q3: 3.9–14.2) months for IMiDs; 4.6 (2.8–6.2) for PIs; 4.4 (2.2–7.5) months for IMiD+PIs. 52% of pts had received one prior line of therapy, most commonly bortezomib-based schemes, and some more than three (8%).

Summary/Conclusion: This observational study represents a snapshot of the management RRMM pts in Italy. Data from a longer follow-up and larger sample size will be presented at the meeting.

C068

EPHA3 PROMOTES ADHESION AND INVASION OF MALIGNANT PLASMA CELLS AND ITS TARGETING REDUCES TUMOR GROWTH *in vivo* IN A MULTIPLE MYELOMA XENOGRAFT MOUSE MODEL

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The tyrosine kinase receptor EphA3 is involved in different processes such as cell viability, cell-cell interaction, cell adhesion and migration. It has been studied in different human malignancies including leukemia but less is known about its presence in MM.

In this study, we analysed the expression of EphA3 by RT-PCR and flow cytometry in plasma cells (PCs) from healthy controls (HCs) and from patients with monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM) and in MM cell lines. Moreover, we also investigated EphA3 role in cell viability by MTS assays, in adhesion to fibronectin (FN) and to bone marrow stromal cells and in invasion by a Matrigel® assay.

We found that EphA3 mRNA and protein are over-expressed in primary MM PCs and in MM cell lines when compared with MGUS patients and HCs (Figure A, B). In addition, loss of function of EphA3 by mRNA silencing significantly inhibited the ability of MM PCs to adhere to FN, to stromal cells and to invade in vitro, without affecting cell proliferation or viability. Gene expression profiling showed that knockdown of EphA3 modulated some molecules that regulate adhesion, migration and invasion processes. Importantly, the treatment with a specific anti-EphA3 monoclonal antibody, significantly inhibited tumor growth in a mouse xenograft MM model.

Our findings suggest that EphA3 plays an important role in the MM biology and provide support that its targeting could represent a novel therapeutic approach in MM.

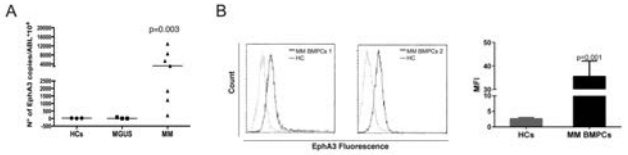

Figure 1.

C069

NUCLEAR LOCALIZATION OF HEME OXYGENASE 1 CONTRIBUTES TO BORTEZOMIB-RESISTANCE IN MYELOMA CELLS

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Background: HO-1 is a cytoprotective microsomal enzyme that catalyzes the degradation of heme. We have recently shown that the protective effect of HO-1 on drug-induced cytotoxicity in leukemic cells does not involve its enzymatic byproducts, but rather its nuclear translocation following proteolytic cleavage. It has been recently described that Bortezomib (BTZ) is able to increase HO-1 expression.

Aims: We investigated about the role of BTZ-induced HO-1 in MM cell lines.

Results: As expected, we observed that Btz (15 nM) induced apoptosis after 24h ($p<0.001$). Flow cytometric analysis revealed increased levels of ROS after 1h ($p<0.0001$) of treatment with a peak after 3h ($p<0.001$). Btz was able to induce a significant increase in HO-1 mRNA levels after 3h ($p<0.0001$) of treatment with a maximum peak after 6h ($p<0.0001$). Since HO-1 is one of the major endoplasmic reticulum (ER) associated heme protein, we analyzed the ability of BTZ to induce ER stress. BTZ was able to induce the expression of ER stress proteins (Bip, IRE1α, Ero1, PERK and CHOP) in MM cells after 6h ($p<0.001$) with a peak after 24h ($p<0.0001$). Moreover, we observed that BTZ was able to induce autophagy-related genes such as ATFG5 and BENC1 in U266 cell lines. Silencing HO-1 using siRNA, we observed reduction of proteins described above indicating that induction of ER stress and then autophagy pathways by BTZ is HO- 1 mediated. Furthermore, by confocal microscope we observed that HO-1 localized both in the cytoplasm and in the nucleus of MM cells. Interestingly, blockage of nuclear translocation by E64, a selective inhibitor of the protein cleavage, induced MM cells to become more sensitive to BTZ ($p<0.001$). No change in cell viability was observed inhibiting HO-1 enzymatic activity by using TIN (zync protoporphirin, 10 uM). Since nuclear HO-1 it has been reported to be a regulator of DNA repair activities, we also explored its role in genomic instability of MM cells. Using the cytochinesis-block micronucleus assay, we observed that pre-treatment of U266 with

E64 for 24h led to a significant reduction of the percentage of micronuclei ($p<0.01$) and nucleolasmic bridges ($p<0.05$) observed in binucleated cells. Next, we evaluated U266 ability to activate G2/M checkpoint after UV damage using cytochinesis block proliferation index assay. The percentage of monucleated cells (G2/M checkpoint activated) was higher in cells pre-treated with E64 than control ($p<0.05$).

Conclusion: Our data suggest that BTZ-induction of HO-1 is probably linked to the activation ER stress and autophagy pathway by BTZ through HO-1 activation. HO-1 nuclear translocation may be involved in MM BTZ resistance. In addition, nuclear HO-1 may be involved in genomic instability of MM cells.

C070

CD38 DEREGULATION AS STRATEGY TO MAKE MULTIPLE MYELOMA CELLS MORE SENSITIVE TO NAD+ DEPLETING AGENTS

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Background. The upregulated NAD+ biosynthesis, which is needed to face increased proliferation and metabolic processes, represents an important feature distinguishing cancer cells from their normal counterparts. As a result, the NAD+ biosynthetic apparatus emerges as highly promising therapeutic target for tumors, as suggested by the use of nicotinamide phosphoribosyltransferase (NAMPT) inhibitors in a number of malignancies, including Multiple Myeloma (MM) and leukemia (Cea *et al*. Blood 2012; Cagnetta et al. Blood 2013; Cagnetta *et al.* CCR 2015). The ecto-enzyme CD38CD38 is emerging as a novel therapeutic target for patients with hematological malignancies, including MM, with several monoclonal antibodies already tested in clinical trials with promising results. Importantly, CD38 by regulating intracellular NAD+ stores acts as member of the intricate network supporting metabolic reprogramming associated with cancer. Based on these assumptions, here we explored CD38 activity as innovative strategy to enhance the anti-tumor activity of NAMPT inhibition in Multiple Myeloma cells.

Methods: A panel of different MM cell lines and primary cells, both sensitive and resistant to conventional and novel anti-MM therapies, was used in the study. The effects of Nampt inhibition was evaluated in presence of CD38-gene editing (loss/gain of approaches) by using CTG assay and Annexin-V/propidium iodide staining. Next, the anti-MM effects of chemicals affecting CD38 activity were also evaluated in combination with low doses of Nampt inhibitors. Mechanistic studies were performed with Western-blotting, lentivirus-mediated shRNAs and enzymatic assays.

Results and Conclusions: By using different approaches, we found that CD38 deregulation makes MM cells more vulnerable to NAD+ depleting agents. Ongoing mechanistic studies suggest the central role played by glucose metabolism in the observed synergism. Overall our data provide the mechanistic preclinical rationale to enhance anti-MM activity of Nampt targeting agents, in order to both overcome drug resistance and improve patients outcome.

C071

IDENTIFICATION OF CLONAL EVOLUTION TRAJECTORIES IN MULTIPLE MYELOMA THROUGH LONGITUDINAL ANALYSIS OF GENOMIC BACKGROUND BY SNPS ARRAY ANALYSIS

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Background. Multiple Myeloma (MM) is a genetically complex disease, whose genetic plasticity favors the coexistence of genetically heterogeneous subclones, selected in a Darwinian fashion throughout the disease course. Therapy might represent a major selective pressure over the different subclones, thus supporting an evolutionary model of the disease.

Aim: To ascertain the existence of clonal evolution patterns in MM, eventually driven by different therapeutic strategies, we longitudinally analyzed a cohort of patients (pts) by using a high throughput technology, able to finely dissect the genetic intra-clonal changes of each pts.

Patients and methods: Fourteen MM pts were included in this study. Pts were up-front treated either with combination regimens including bortezomib (9 pts) or with conventional chemotherapy (5 pts). For each pts, paired bone marrow (BM) samples were collected both at diagnosis and at relapse. SNPs array analyses were performed on the BM CD138+ cell fractions (6.0 and CytoscanHD Affymetrix arrays) and data were analyzed with ChAS v3.1 and Nexus software, to obtain Copy Number Alterations (CNAs) results.

Results: Overall, 5839 and 8308 CNAs were observed in CD138+ plasma cells collected from newly diagnosed and relapsed pts, respectively: indeed, the frequency of both CN gains and CN losses was higher in samples collected at relapse (20% CN gains and 24% CN losses) as compared to those collected at diagnosis (14% CN gains and 0,7% CN losses). The frequency of CNAs observed in 27 genes of interest (selected according to their recognized role in MM pathogenesis) was analyzed in details: overall, very few changes were highlighted in the frequencies of clonal alterations. On the contrary, by comparing the frequencies of sub-clonal alterations observed in the same 27 genes, several changes were shown. Accordingly, three major patterns were highlighted. In 5/14 (36%) pts, no changes in the genomic background were observed at relapse, as compared to diagnosis. In 3/14 (21%) pts, increased frequencies of several CNAs were observed in samples collected at relapse, as compared to those collected at diagnosis. Finally, in 6/14 (43%) pts, either increased or decreased frequencies of several CNAs were observed in samples collected at relapse, as compared to those collected at diagnosis. Interestingly, pts showing no changes in their genomic background have been up-front treated with conventional chemotherapy, whereas pts showing changes, with bortezomib-based regimens.

Conclusions: By employing SNPs array-based technology to explore the genomic background, a sub-clonal dynamics over time was confirmed in MM pts. The emergence of changes at relapse, with respect to diagnosis, suggests the existence of either a linear or a branching evolution of the subclones present at the onset of the disease, possibly driven by the selective pressure exerted by the therapeutic treatment.

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C072

DISSECTION OF THE IMMATURE STATE AND IMMUNOPHENOTYPIC PLASTICITY OF MM CLONE(S) AT DIAGNOSIS BY MULTIPARAMETRIC FLOW CITOMETRY ANALYSIS

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Introduction: The sequence of events underlying the process of Multiple Myeloma (MM) plasma cells (PC) differentiation have not yet fully elucidated, even if recent findings suggest that different cell subpopulations, with distinct phenotype, compose the MM clone(s), whose plasticity has emerged as a typical feature. The phenotypic states' transition has been described both as an intrinsic biological characteristic and as a trait tuned in response to extrinsic factors (e.g. therapy).

Aim: To evaluate the phenotypic plasticity of MM PC at diagnosis in order to stratify patients (pts) according to the PC differentiation stages and to evaluate the impact of this stratification on the disease outcome.

Patients and Methods: 24 newly diagnosed MM pts (16 symptomatic and 8 asymptomatic, as defined according to the IMGW criteria) were included in this study. Phenotypic characterization was carried out on fresh BM samples, by 6-color multiparametric flow citometry analysis, combining CD138-PE, CD38-PE-Cy7, CD20-APC, CD19-APC-Cy7, CD27-FITC, CD45-FITC, CD28-APC, CD44-FITC, CD54-APC, CD81-PerCP-Cy5.5 and CD56-APC (Miltenyi Biotech, Beckton Dickinson). The co-expression of both CD19 and CD81 in the same pts was inferred by overlapping the Median Fluorescence Intensity (MFI) peaks of these antigens (BD FACS Canto II; Diva Software v6.1.3).

Results: In each pts, neoplastic PC were recognised by the expression of CD56, which resulted quite variable among pts, with higher median frequencies in symptomatic as compared to asymptomatic pts (88,7% vs. 66,8%). Overall, symptomatic pts carried a higher PC fraction at the disease onset, with respect to asymptomatic ones (9,2% vs. 5,6%). Interestingly, only 9/24 pts displayed a conventional MM phenotype (as described according to EuroFlow guidelines), whereas 15/24 pts showed an aberrant PC phenotype, with highly heterogeneous expression of the analysed phenotypic markers, thus suggesting a high grade of plasticity of the MM clone. To better define the PC differentiation stages, the CD19/CD81 co-expression was evaluated for each pts, thus allowing the identification of three sub-groups, whose PCs were either CD19+/CD81+, or CD19-/CD81+, or CD19-/CD81- (12, 4 and 8 pts, respectively). The less mature CD19+/CD81+ differentiation stage was confirmed by both the high CD20 expression (51,60% vs. 33,8%), frequently expressed by more clonogenic and primitive MM cell populations, and the low CD28 expression (2,58% vs. 17,94%), known to be a key mediator of MM survival and apoptotic resistance. Pts carrying CD19+/CD81+ PC were finally characterized by a higher median free light chain ratio, as compared to pts carrying more mature PC (246,63 vs. 49,90).

Conclusions: MM clone(s) is a mixture of different cell populations endowed with an inner phenotypic plasticity. Various PC differentiation stages were appreciable already at diagnosis and clinical features associated to bad prognosis characterize pts carrying more immature clones.

Acute Leukemia 2

C073

DOT1L INHIBITOR EPZ-5676 SYNERGIZES WITH SORAFENIB IN PRECLINICAL MODELS OF PEDIATRIC ACUTE MYELOID LEUKEAMIA (AML)

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Background: MLL gene rearrangements (MLL-r) account for about 20% of the childhood AML, including ~80% of infant acute leukemias. MLL-r AMLs have a particularly poor prognosis, and thus new therapeutic approaches are urgently needed. Because abnormal expression of MLL-r target genes is associated with high levels of H3K79 methylation, novel promising inhibitors of the methyltransferase DOT1L entered in clinical trials. MLL-r also showed a cooperative effect with activated FLT3. Indeed, FLT3 inhibitors, including Sorafenib, demonstrated encouraging efficacy in AML.

Aim: To investigate the efficacy of a combination treatment using DOT1L inhibitor EPZ-5676 and Sorafenib to treat MLL-r AML.

Methods. MLL-r (MOLM13, NOMO1, MV4-11, THP1) and non MLL-r (OCI-AML3, HL60, U937) AML cell lines, and MLL-r primary samples from pediatric AML patients were used. Flow cytometry analyses were performed to assess absolute cell counting and apoptosis. Protein expression and H3K79me2 were quantified by Western blot. mRNA expression was studied by quantitative Real-Time PCR.

Results: Treatment of AML cells with increasing concentrations of EPZ-5676 up to 16 days demonstrated a significant cell growth inhibition in several MLL-r (MOLM13, NOMO1, MV4-11) as well as in non MLL-r OCI-AML3 cells, so that the impact of DOT1L inhibition could not exclusively rely on MLL-r. However, a significant apoptosis occurred only in MOLM13 and MV4-11, thus suggesting that DOT1L inhibition alone might not be able to induce cytotoxicity. Repression of DOT1L occurred since the 4th day of treatment, as demonstrated by the loss of H3K79me2. To further explore the consequence of this phenomenon, both MLL targets and key component of signaling pathways involved in AML survival (i.e. PI3K, FLT3 and MAPK) were investigated. Gene expression of HOXA9, MEIS1 and FLT3 decreased in all cell lines, whereas STAT5 and c-Myc mRNAs, along with STAT5 protein expression, were downregulated only in MLL-r cells. In MOLM13 and NOMO1 cells p-Erk was strongly reduced, whereas a strong induction in p-S6RP was observed in U937 cells after prolonged treatment, suggesting the possible involvement of PI3K pathway in drug resistance mechanisms. To increase the benefit of DOT1L inhibition, both AML cell lines and MLL-r primary samples were pretreated with increasing concentration of EPZ-5676 for 4/8 days, following 24/48h treatment with Sorafenib. This combination resulted in a synergistic effect in 4/7 cell lines and irrespective of MLL-r. Moreover, in primary AML samples both EPZ-5676 and Sorafenib showed a limited effect as single agent, whereas their combination increased apoptosis.

Conclusion: These results demonstrated that EPZ-5676 has a limited antileukemic activity, which is not restricted to MLL-r AMLs. However, the combination of EPZ-5676 with Sorafenib revealed a synergistic effect in both MLL-r and non MLL-r AMLs, paving the way to innovative and more effective treatments for pediatric AML patients.

C074

TITLE: NOTCH SIGNALLING INHIBITION AS A MULTI-TARGET THERAPY TO OVERCOME BONE MARROW STROMAL MICROENVIRONMENT-MEDIATED CHEMORESISTANCE IN ACUTE MYELOID LEUKEMIA (AML) CELLS

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Background and aims: Notch signalling is a master developmental pathway that controls tumourcell survival by interacting with pro-survival proteins, such as β-catenin, BCL-2,Stat3, NFκB, and AKT, thus representing an ideal target to interfere with all these pathways in different cancer systems. We recently showed that Notch inhibition was capable of abrogating microenvironment-mediated AML cell chemoresistance(Oncotarget 2016, in press; doi: 10.18632/oncotarget.7964); in this study, we analyzed the mechanisms underlying microenvironmental, Notch-mediated AML chemoresistance by investigating the contribution of BCl-2, STAT3, NFκB and AKT.

Methods: Using in silico and in vitro approaches we analyzed the expression changes of prosurvival proteins in ex-vivo AML cell samples in condition of pharmacological or genetical Notch down-regulation, as well as in AML cells either cultured alone or co-cultured with human bone marrow mesenchymal stromal cells (hBM-MSCs) in presence of chemoterapeutic agents, such as cytarabine (Ara-C) and Idarabicin.

Results: In silico Gene set enrichment analysis and flow cytometry analysis showed that AML samples highly expressed Notch1, Jagged1, STAT3, NFκ-B and AKT genes and proteins. Notably, higher levels of Notch1 were found in patients with poor cytogenetic prognosis, while STAT3, NF-κB and AKT were uniformly expressed by AML patients. Protein analysis revealed low levels of pro-survival proteins AKT, STAT3 and NF-κB in RBP-jk and MALM1 knock-down cells,as compared to control cells infected with non specific shRNA. We then verified that genetic (shRNA) and pharmacological inhibition of Notch,by using either GSIs or Notch receptor blocking antibodies, was capable of sensitizing AML cells, either cultured alone or in presence of hBM-MSCs, to ARA-C or Idarubicine. Additionally, we found that hBM-MSC-dependent induction of AML chemoresistance was associated to increase of AKT, NF-κB and STAT3 protein level in AML cells. Similarly, Notch inhibition with GSIs prevented hBM-MSC-mediated increase of AKT, NF-κB and STAT3, thus restoring sensibility of AML cells to Idarubicin treatment.

Conclusion: These results suggest that inhibition of Notch signalling is sufficient to reduce protein levels of AKT, STAT3 and NF-κB proteins involved in AML chemoresistance, thus making the pro-survival core network controlled by Notch a potential target for specific therapies.

C075

RATIONAL OF TARGETING WNT/β-CATENIN SIGNALLING IN ACUTE MYELOID LEUKEMIA (AML)

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Background and aim: Although recent evidence suggests that aberrant Wnt signaling can be involved in the neoplastic myeloid cell growth, the role of the Wnt/β-catenin pathway during the disease development and its contribution to AML chemoresistance are still unclear.

Methods: AML primary blast cells(25 samples) and AML cell lines were cultured or co-cultured with human bone marrow mesenchymal stromal cells (hBM-MSCs),in presence or absence of Wnt modulators, including ligands (Wnt3a, Wnt5a/5b), Porcupine inhibitors (IWP-2), LRP6 inhibitors (Niclosamide), or antagonists of TCF/β-catenin (PKF118-310, PNU-74654).

Results:In silico analysis showed the enrichment of Wnt signaling components in AML samples. Western Blot and flow cytometry showed the presence of total β-catenin only in 65% of primary samples analyzed. β-catenin positive samples had different degree of activation of the pathway, as revealed by the expression of active forms of β-catenin, including (Ser675)β-catenin and non-phospho-(Ser33/37/Thr41) β-catenin. Notably, we found that active forms of β-catenin increased in AML samples in co-culture with hBM-MSCs, thus suggesting that Wnt signalling could be involved in the crosstalk between bone marrow stroma and AML cells. The addition of Wnt pharmacological inhibitors,such as IWP-2, PNU-74654 and Niclosamide,to the culture medium of β-catenin-positive AML samples, either cultured alone or in co-culture with hBM-MSCs, reduced AML cell proliferation with slight effect on cell death. When associated to Idarubicin,all Wnt inhibitors except IWP-2 synergycally induced a dramatic cell death in AML cells in both culture conditions. However, when Idarubicin was replaced by Ara-C the synergism was observed only with Niclosamide and PKF. Cell death was mainly due to apoptosis, as shown by Annexin-V staining.

Conclusion: Overall our data show that chemoterapeutic agents in combination with Wnt inhibitors reduce proliferation and chemoresistance of AML cellsin culture or co-culture with bone marrow stroma, thus highlighting the role of microenvironmental Wnt/β-catenin pathway in supporting AML cell survival. Consequently, Wnt/β-catenin signalling may represent a therapeutic target to enhance the effectiveness of chemotherapeutic agents in AML.

C076

DIASORIN Q-LAMP IS A RELIABLE, ROBUST AND ULTRARAPID MOLECULAR TEST FOR PML-RARA TRANSCRIPTS IDENTIFICATION EVEN IN CASE OF DEGRADATED, INHIBITED AND LOW CONCENTRATED RNA SAMPLES

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Background: the availability of ultra-rapid screening tests, easy to be performed and robust even in case of low quality RNA samples, can significantly enhance the management of patient affected by APL, allowing rapid initiation of therapy.

Methods: the DiaSorin Q-LAMP PML-RARA assay is a fluorescent multiplex method that allows the simultaneous differential detection of the bcr1/bcr2/bcr3 PML-RARA isoforms. It amplifies starting directly from RNA under isothermal conditions onto the Liaison Iam instrument (DiaSorin), a small footprint device that incubates the reactions for 40 minutes at constant temperature, monitoring in real time the amplification signals. Thanks to the one-step format, the internal control amplification (specific for the endogenous GUSB mRNA) and the great robustness of the technology, the risks of false or inconclusive results is highly reduced. To evaluate the technology reliability and robustness, 166 RNA samples (128 PML-RARA positive by RT-PCR and 38 negative) were tested. Among these, fifty three samples (33 PML-RARA positive and 20 negative) were interested by heavy chemical contamination. In addition we analyzed by Q-LAMP seven samples that couldn't be amplified by RT-PCR because of strong RNA degradation (n=3), inhibition (n=2) and very low RNA amount availability (n=2) and six samples that didn't produce an acceptable amplification result by RT-PCR (bcr1/bcr2 undefined) and which results have been confirmed by direct sequencing.

Results: The DiaSorin Q-LAMP PML-RARA demonstrated 100% concordance with RT-PCR on all clinical samples, including the 53 poor quality RNAs. The additional 7 samples for which RT-PCR analysis was unsuccessful, were all effectively amplified by Q-LAMP, demonstrating a very high robustness. Moreover, when the test was carried out on samples with a bcr1/2 uncertain transcript using RT-PCR, the Q-LAMP was able to discriminate with high accuracy the correct isoform, as proven by sequencing results. All positive samples were amplified in less than 30 minutes, while the negative samples were validated by the amplification of the housekeeping GUSB mRNA.

Conclusions: The DiaSorin Q-LAMP method demonstrated an improved robustness over RT-PCR especially on low concentrated, contaminated and degraded samples. This feature united with the speed, the closed tube format and the ready-to-use lyophilized reagents results into a simple, safe and reliable solution that eliminates the need for analysis repetition or sample re-extraction. In conclusion, the Q-LAMP solution can be a powerful tool for improving the diagnostic work-up of patients affected by APL.

C077

SIMPLIFIED MOLECULAR SCREENING OF CBF-AML BY THE DIASORIN Q-LAMP TECHNOLOGY

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Introduction: The t(8;21) and the inv(16)/t(16;16) are chromosomal aberrations characteristic of Core-Binding Factor Acute Myeloid Leukemias (CBF-AML), a subclass of AML with a favourable prognosis. The derived fusion transcripts (AML1-ETO and CBFB-MYH11 respectively) are therefore routinely tested by RT-PCR during the diagnostic work-up of AML with the purpose of risk stratification.

Methods: Here we present a novel fluorescent Q-LAMP assay panel developed by DiaSorin aimed to simplify and speed-up the molecular detection of the AML1-ETO and CBFB-MYH11 fusion transcripts in AML patients at diagnosis. The system consists in one duplex assay specific for the AML1-ETO transcript and in two multiplex assays specific for the most common CBFB-MYH11 transcripts (type A and types D/E, respectively). The assays simultaneously detect also the endogenous GUSb mRNA as an internal control for the validation of negative samples. Both AML1-ETO and CBFB-MYH11 Q-LAMP assays are isothermal amplifications that start directly from RNA samples, in a one step and closed format. Results can be obtained in less than 30 min and can be monitored in real time. In this study we evaluated the analytical sensitivity of Q-LAMP assays using AML cell lines positive for the specific translocation. Moreover, the RNA obtained from 106 healthy donors and from 83 CBF-AML patients (39 positive for AML1-ETO and 44 positive for CBFB-MYH11), previously analysed by RT-PCR (Biomed protocol) were also tested by Q-LAMP.

Results: The analytical sensitivity has been determined by testing serial dilutions of mutated sequences (RNA from Kasumi-1 cell line positive for AML1-ETO, RNA from ME-1 cell line positive for CBFB MYH11 type A and plasmids carrying the CBFB-MYH11 type D and E sequences) into the RNA from HL-60 cell line (negative for the translocation of interest). Dilutions containing the AML1-ETO transcript at 10^{-4} level have been 100% detected. For the CBFB-MYH11 type A, the 10^{-3} and the 10^{-4} ME-1 dilutions have been detected in 100% and 73.2% of replicates, respectively. Dilutions of 40 copies of plasmids D and E showed a 97.8% and 100% detection, respectively. Moreover, all the RNA clinical samples defined CBF-AML positive by RT-PCR and negative RNA samples obtained from healthy donors have been correctly detected by Q-LAMP (100% concordance with the expected results).

Conclusions: the AML1-ETO and CBFB-MYH11 Q-LAMP-based panel developed by DiaSorin represents a convenient solution for the molecular screening of CBF-AML at diagnosis. The closed-tube format simplifies the entire procedure, fastening the set-up and decreasing risk of errors and contamination classically associated to the multi-steps PCR procedures. The multiplex format, monitorable in real time, and the ultra rapid performance optimizes time and resources, allowing easy implementation across all type of laboratories.

C078

THERAPY-RELATED MYELOID NEOPLASMS REPRESENT A DISTINT EPIGENETIC ENTITY WITH DEREGULATION OF KEY BIOLOGICAL PATHWAYS

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Introduction: Therapy-related myeloid neoplasms (t-MN), including AML and MDS, represent distinct clinical and genetic entities, defined by a previous cytotoxic treatment for a primary disease. Both the type of therapeutic agent used for primary malignancies treatment and the type of primary neoplasm have been shown to contribute to the molecular makeup of the t-MN. Epigenetic deregulation is a key process that contributes to the development of myeloid malignancies, and mutations in epigenetic modifiers can be found at high frequencies in most of these malignancies. Whilst it is well known that abnormal methylation patterns are a hallmark of de novo MDS and AML, little is still known about epigenetic deregulation in t-MN. In order to address this and gain better insight into the epigenetic deregulation of t-MN, we performed comprehensive DNA methylation profiling of a cohort of t-MN using next-generation bisulfite sequencing and compared our findings to the profiles observed in non-therapy related myeloid malignancies.

Methods: The patient cohort included 25 patients with a diagnosis of t-MN (n=15), de novo MDS (n=5) or acute myeloid leukemia (AML) secondary to a myeloproliferative neoplasm (sec-AML; n=5). According to primary neoplasm, 5 out of 15 t-MN patients had a prior history of Hodgkin lymphoma, 5 had a prior history of non-Hodgkin lymphoma and finally 5 had a prior history of breast cancer. DNA was extracted from BM-MNCs at the time of initial diagnosis. The Enhanced Reduced Representation Bisulfite Sequencing (ERRBS) assay was used to quantified cytosine methylation at ~3M CpG sites across the genome and the single-locus quantitative bisulfite sequencing by MassARRAY EpiTYPER was used to validate ERRBS data.

Results: Methylation profiles did not differ according to blast proportion in t-MDS and t-AML. When compared to their de novo counterpart, t-MDS showed a predominant loss of DNA methylation, with 83% of the differentially methylated regions (DMRs) being hypomethylated. DMRs were significantly depleted from promoter regions, and were enriched at distal intergenic regions, particularly at enhancer loci. In t-AML DMRs were also hypomethylated compared to AML evolving from MPN and they were significantly depleted at promoter regions and CpG islands, but enriched at exonic regions. Both in t-MDS and t-AML, DMRs belonging to WNT and cadherin signaling pathways genes were highly overrepresented. When grouping patients according to the primary tumor, we found that methylation levels were lower in t-MN secondary to lymphomas, compared to breast cancer. In particular, in the lymphoma subset, Hodgkin lymphomas were characterized by significant hypomethylation, compared to non-Hodgkin lymphomas.

Conclusion: Our data show that t-MN are characterized by a hypomethylated phenotype, with significant differences according to the type of primary neoplasm.

C079

MYC-CONTAINING DOUBLE MINUTE CHROMOSOMES IN AML: ORIGIN, STRUCTURE AND TRANSCRIPTIONAL FEATURES

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Introduction: We have previously demonstrated that the loop formation-excision-amplification (episome) model is involved in the genesis of MYC (8q24) amplifications as double minutes (dmin) in AML. Despite the origin of dmin was recently explained through a single-event of chromosome shattering (chromothripsis), our results excluded this mechanism in HL-60 and solid tumor cell lines with MYC-dmin. In addition, the finding that MYC is not over-expressed when amplified in AML suggested a non-linear correlation between gene amplification and over-expression.

Methods: By integrating whole genome sequencing (WGS, Illumina Xten), SNP array (Affymetrix 6.0), whole-transcriptome sequencing (Illumina HiSeq2500), FISH and PCR techniques, we: 1) reconstructed the internal structure of head-to-tail amplicons in 23 AML cases with MYC-dmin; 2) performed chromothripsis analysis, according to established criteria; 3) identified chimeras involving amplified genes as either 5' or 3' partners; 4) find out genomic rearrangements supporting fusion transcripts; 5) compared the gene expression profile (GEP) of AML-dmin to AML with normal karyotype.

Results: 1) Mainly, a single 8q24 amplicon was identified on dmin. In few cases, we found coexisting heterogeneous amplicons, differing in complexity and chromosome content; 2) our bioinformatics analyses led us to exclude chromothripsis as the driving force underlying amplicon genesis in our samples. Moreover, in 55% of cases, the amplified region was deleted in one of the chromosome 8 homologs, suggesting the excision of a DNA segment from the original chromosomal location; 3) multiple 8q24 chimeras were validated by RT-PCR and Sanger sequencing, showing the involvement of PVT1 (as either 5' or 3' partner), MYC (3'), FAM49B (5' or 3') and TRIB1 (3'). Interestingly, some of them were recurrent or present as multiple transcript isoforms or occurred together with their reciprocal fusion transcript. Notably, none of them was observed in AML with normal karyotype; (4) WGS disclosed the lack of genomic rearrangements supporting such chimeras; (5) the differential GEP analysis showed the up-regulation of many amplified genes, although MYC displayed an inconsistent expression level among patients. Interestingly, PCA showed a clear separation between AML-dmin and AML with normal karyotype.

Conclusions: Our data excluded chromothripsis in the genesis of MYC-dmin in AML. Hence, the episome model should be reconsidered as still valid to describe dmin emergence. Moreover, dmin were accompanied by the occurrence of novel 8q24 fusion transcripts not corresponding to genomic rearrangements, suggesting the involvement of post-transcriptional events that could lead to a dramatic transcriptome remodeling. In this view, a possible impact upon MYC expression pattern will need further clarifications.

C080

EPIGENETIC REGULATION OF UNCX, A HOMEOBOX TRANSCRIPTION FACTOR GENE ECTOPICALLY ACTIVATED IN ACUTE MYELOID LEUKEMIA

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Introduction: Deregulation of the homeobox (HB) gene Uncx, which is embedded within a low-methylation (‚â§ 10%) region (canyon), was epigenetically induced by altered DNA methylation at the borders of the gene canyon due to Dnmt3a inactivation in murine hematopoietic stem cells (HSCs). HB genes alterations are often correlated with leukemogenesis, although the pathogenic significance of these changes is poorly understood. UNCX (7p22.3), encoding for a transcription factor involved in somitogenesis and neurogenesis, is specifically expressed in eye, brain and kidney. It is never reported as associated with human cancer.

Methods: RT-qPCR was carried out to quantify UNCX expression level in a cohort of acute myeloid leukemia (AML) patients and cell lines, including one case with a t(7;10)(p22;p14) translocation. The functional effects linked to UNCX ectopic expression in myeloid cells were evaluated by retrovirus-mediated UNCX transfer in purified cord blood CD34+-derived cells. Gene Expression Profiling (GEP) by Exon Array was performed to identify a possible transcriptome signature of UNCX-positive versus UNCX-negative patients. These results were compared with the GEP obtained by RNA sequencing datasets of 170 AML patients available at The Cancer Genome Atlas (TCGA) database and correlated with patient survival. Moreover, DNA methylation ratios of the UNCX canyon and DNMT3A mutational status were assessed through Mass Array Sequenom and NGS technologies, respectively.

Results: We observed UNCX ectopic activation, possibly due to a position effect, in the patient harbouring a t(7;10) translocation showing a breakpoint upstream of this gene, as well as in 32.3% (20/62) of AML patients and 8% (6/75) of AML cell lines without the rearrangement. *in vitro* studies on UNCX-transduced wild-type CD34+ HSCs indicated a clear role of UNCX in slowing myeloid cell proliferation and differentiation, which was supported by gene-expression profiling of primary cells. Notably, UNCX expression, associated with a marked downregulation of the proliferation marker MAP2K1 and upregulation of CCNA1, impaired differentiation and maintained pluripotency of infected myeloid cells versus controls. The comparison between the GEP from Exon array and TCGA database confirmed deregulation of genes involved in myeloid cell proliferation, survival, and differentiation (MPO, CCNA1, MAP2K1, and MAFB). UNCX activation was associated with significant methylation changes at canyon edges in patients, but was uncorrelated with DNMT3A mutations. Notably, UNCX-positive AML patients showed a trend toward diminished overall survival.

Conclusions: Here, we describe a novel leukemogenic role in AML for the HB gene UNCX, whose activation is induced by epigenetic modifications, although not correlated with DNMT3A mutations. Our findings provide new insights into the role of epigenetically activated HB genes in leukemogenesis and suggest a potential prognostic value for UNCX expression.

Molecular Hematology

C081

LIQUID BIOPSY AS A TOOL FOR MONITORING THE GENOTYPE OF DIFFUSE LARGE B-CELL LYMPHOMA

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Introduction: Accessible and real-time genotyping for diagnostic, prognostic or treatment purposes is increasingly impelling in diffuse large B-cell lymphoma (DLBCL). Since DLBCL lacks a leukemic phase, tumor genotyping has so far relied on the analysis of the diagnostic tissue biopsy. Cell-free DNA (cfDNA) is shed into the blood by tumor cells undergoing apoptosis and can be used as source of tumor DNA for the identification of somatic mutations. cfDNA is representative of the entire tumor heterogeneity, thus allowing to identify mutations from tumor cells residing in non-biopsied sites. Also, serial sampling of plasma cfDNA allows to track the emergence of targeted drug-resistance mutations. Here we aimed at tracking the DLBCL genetic profile using plasma cfDNA.

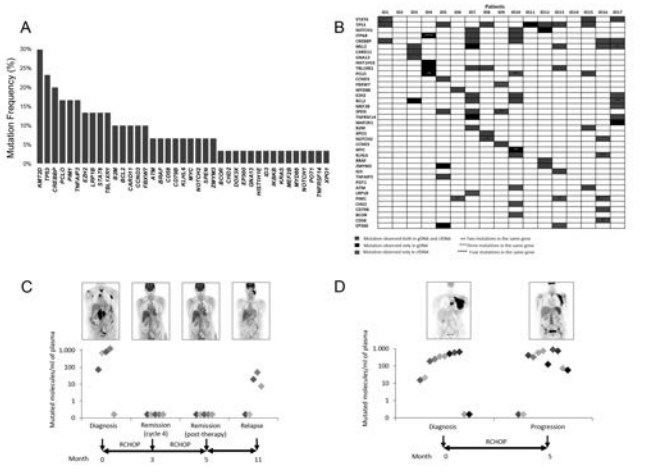

Figure 1.

Methods: The study includes 30 DLBCL patients provided with cfDNA from plasma collected at diagnosis, during R-CHOP course, at the end of treatment and at progression. Paired normal genomic DNA was collected for comparative purposes to filter out polymorphisms. Paired tumor genomic DNA from the diagnostic tissue biopsy was available for 26 patients (17 fresh/frozen, 9 FFPE). A targeted resequencing panel including the coding exons and splice sites of 59 genes that are recurrently mutated in mature B-cell tumors was designed. Ultra-deep NGS was performed on MiSeq (Illumina). RESULTS. Pretreatment cfDNA genotyping disclosed somatic mutations in known DLBCL-associated genes, including KMT2D (30%), TP53 (23%), CREBBP (20%), PIM1 and TNFAIP3 (16%), EZH2, TBL1XR1 and STAT6 (13%), B2M, BCL2, CARD11, CCND3, FBXW7 (10%) (Figure 1A). Genotypes from cfDNA and paired tumor genomic DNA (gold standard) were compared to derive the diagnostic accuracy of cfDNA analysis (Figure 1B). Genotyping of the paired plasma cfDNA correctly identified 79% of the biopsy-confirmed mutations. ROC analysis showed that cfDNA genotyping had the highest sensitivity (96%) in discovering mutations that were represented in >20% of the alleles of the tumor biopsy. Longitudinal analysis of plasma samples under R-CHOP chemotherapy showed a clearance of the DLBCL mutations in the cfDNA among responding patients (Figure 1C). Among patients resistant to R-CHOP, basal DLBCL mutations did not disappear from cfDNA. In addition, among treatment-resistant patients, new mutations were acquired in cfDNA that marked resistant clones selected during the clonal evolution (Figure 1D). CONCLUSIONS. These results demonstrate that cfDNA genotyping of DLBCL: i) is as accurate as genotyping of the diagnostic biopsy to detect somatic mutations of allelic abundance >20%; and ii) is a real-time and non-invasive way to track clonal evolution and emergence of treatment resistant clones.

C082

MONOSOMY 7 AS CLONAL EVOLUTION OF UNSTABLE CHROMOSOME 7 DERIVATIVES

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Introduction: Monosomy of chromosome 7 (-7) is one of most frequent aneuploidies in AML and MDS and it is associated with adverse outcome. It can be frequently found as sole anomaly or within complex karyotype in secondary AML or MDS. The mechanism leading to the loss of whole chromosome 7 (chr.7) has not been clarified. In patients with aplastic anaemia, a recent study has shown a correlation between telomeres shortening and progression to AML, frequently associated with acquisition of -7. As telomeres are essential in preserving chromosome integrity, it is possible to speculate that loss of telomeres on chr.7 might result in an unstable derivative 7 (der(7)) that is lost during cell division. We performed a retrospective study to evaluate the presence of clonal evolution involving chr.7. METHODS: FISH analysis with specific probes for chr.7 was performed in 39 AML/MDS patients showing by conventional cytogenetic (CC): 1) several clones with different chr.7 abnormalities; 2) unidentified marker chromosomes and -7 in the same clone or in different clones; 3) dicentric chromosomes. RESULTS: Sixteen cases showed different clones with different chr.7 abnormalities. By CC, most patients (n=9) showed two clones: one clone with -7 and another one with -7 and a marker chromosome. FISH identified the marker as der(7) (Figure 1), probably a ring chromosome, with deletion of 7q31 region (del(7q)) in 8 cases and deletion of 7p arm (del(7p)) in one case. Other 2 cases showed del(7p) in one clone and -7 in another clone. Three cases showed 3 different clones by CC: one with del(7q), one with additional material on 7q and the last one with -7. In one case, we observed a clonal evolution starting from del(7q) within non-complex karyotype, followed by the emergence of a clone with additional material on 7q within complex karyotype and finally the emergence of the clone with -7. In one case, a weak signal for chr.7 centromere was detected on a small chromosome fragment, while 10% of interphase nuclei showed complete -7. In last case, showing dicentric chromosome, FISH showed complete -7 in 6% of cells. In 4 cases, the signal of the centromeric region on der(7) was weaker than the one on the normal chr.7 suggesting partial loss of centromeric sequences. Eight patients were previously exposed to genotoxic agents while the remaining patients were diagnosed as de novo MDS (n=4) or AML (n=4). Three MDS patients showed rapid progression to AML associated with increasing of -7 clone size. Five patients showed RPN1/MECOM rearrangement. CONCLUSIONS: These data suggested that, at least in some cases, -7 is the end-stage of clonal evolution starting from structural rearrangements of chr.7. The loss of

centromeric sequences on der(7) could destabilize the link between centromere and kinetochore and explain its proneness to be lost during mitosis. Further analysis to establish telomeres length and the role of telomeres maintaining proteins are planned.

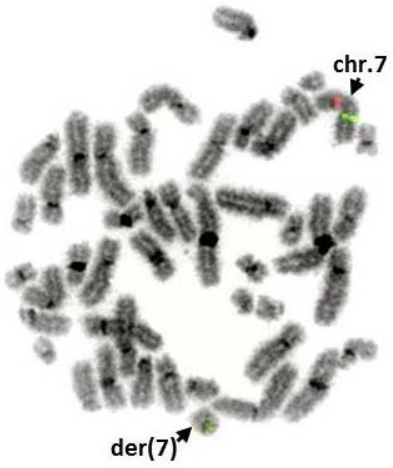

Figure 1.

C083

MYD88L265P MUTATION DETECTION IN WALDENSTR√ÑM MACROGLOBULINEMIA: MINIMAL RESIDUAL DISEASE MONITORING AND CHARACTERIZATION ON CIRCULATING FREE DNA BY DROPLET DIGITAL PCR

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Background: MYD88-L265P mutation in Waldenstr√∂m Macroglobulinemia (WM) represents an ideal marker for minimal residual disease (MRD) monitoring. However, current tools, as allele-specific quantitative PCR (ASqPCR), are not sensitive enough for MRD detection on samples harboring low concentrations of circulating tumor cells as peripheral blood (PB) or cell-free DNA (cfDNA).

Aims: Here we describe the feasibility of a new, highly sensitive, ddPCR assay for MYD88-L265P detection and MRD monitoring in bone marrow (BM), PB and cfDNA from plasma and we compared this strategy with the standard IGH-based MRD approach.

Methods: BM, PB and plasma samples from 68 WM, 6 IgG-lymphoplasmacytic lymphomas(LPL) and 3 IgM-MGUS patients (pts) were collected at baseline and during follow-up. 20 healthy subjects were used as negative controls. MYD88-L265P was assessed on gDNA and cfDNA by a custom ddPCR assay on a QX100 System (Bio-Rad). For comparison ASqPCR was assessed on gDNA, as described [Xu L, 2013]. MYD88-L265P cut-off was settled based on the healthy samples background level. Moreover, IGH-based MRD analysis was performed as described [Ladetto M, 2000; Drandi D, 2015].

Results: Once optimized, MYD88-L265P ddPCR assay sensitivity was compared to ASqPCR. Whereas ASqPCR confirmed the reported sensitivity of 1.00E–03, ddPCR reached a sensitivity of 5.00E–05. Overall, 137 samples from 77 pts (68 WM, 6 LPL, 3 IgM-MGUS), 86 baseline (64 BM, 22 PB) and 51 follow-up (23 BM and 28 PB), were analyzed. 63/64 (98.4%) diagnostic BM and 19/22 (86.4%) PB samples scored positive for MYD88-L265P (BM median 4.5%, range: 0.02-72.6%: PB median 0.15%, range: 0.01-27.8%) (all 3 negative PB had a positive BM match). Concordance between methods was investigate on 100 samples (60 BM, 40 PB) and overall a good concordance was observed (p=0.0005). Of note the majority of discordances were observed in the follow-up samples (13/60 ddPCR positive/AsqPCR negative, 11/60 ddPCR negative/AsqPCR positive). Moreover, to investigate whether MYD88-L265P ddPCR could be used for MRD monitoring we compared it to the gold standard IGH-based MRD assay. From 33/57 (57.9%) pts displaying an IGH rearrangement, baseline and follow-up samples from 10 preliminary pts (23 BM, 15 PB) were analyzed, showing highly superimposable results between methods. Finally, pivotal results on cfDNA from 33 pts showed 1 log higher median levels of MYD88-L265P mutation in plasma (0.7%, range 0-25,7%) compared to PB (0.037%, range: 0.01-20.0%).

Conclusions: MYD88-L265P ddPCR is a feasible and highly sensitive assay for screening and MRD monitoring in WM, particularly in samples harboring low concentrations of circulating tumor cells. Moreover, cfDNA from plasma represents a promising tissue source and might be an attractive less invasive alternative to PB or BM for MYD88-L265P detection. Methodological validation against IGH-based MRD detection and flow cytometry, as well as correlations with clinical data, are currently ongoing.

C084

A 6-GENE EXPRESSION SIGNATURE IN MANTLE CELL LYMPHOMA: RESULTS FROM THE FONDAZIONE ITALIANA LINFOMI (FIL)-MCL-0208 TRIAL

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Given the clinical heterogeneity of mantle cell lymphoma (MCL), we aimed to identify subsets with peculiar biological features in the context of a cohort of homogeneously treated MCL patients. The study used gene expression profiling (GEP) and quantitative real-time PCR (qRT-PCR) validations in peripheral blood (PB, n=46) and formalin fixed paraffin embedded (FFPE, n=42) samples from 82 MCL cases enrolled in the Fondazione Italiana Linfomi (FIL)-MCL-0208 trial (high-dose therapy followed by autologous transplantation). Results: i) Unsupervised and supervised analyses. GEP from 27 PB samples were analyzed by principal component analysis (PCA) and divided in two subgroups PCA1 (14 cases) and PCA2 (13 cases). Supervised analysis, according to PCA classification, identified a gene expression signature of 902 probes (700 up-regulated). Gene Set Enrichment Analysis (GSEA) demonstrated a significant enrichment of five B Cell Receptor (BCR)-related gene

sets, being constitutively over-expressed in PCA2 samples. ii) Identification of a "PCA2-type" gene signature. By merging the lists of differentially expressed genes, a group of 14, PCA2 overexpressed genes was obtained. Six genes, AKT3, BCL2, BTK, CD79B, PIK3CD, and SYK, were selected for further validations. iii) Generation of a 6-gene prediction model. These 6 genes were analyzed by qRT-PCR to generate a prediction model by using 17 PB cases as training cohort and 10 cases as validation cohort. 10/10 cases of the validation cohort were correctly assigned according to the PCA2/PCA1 classification. qRT-PCR was then utilized to classify 19 additional cases (10 PCA2 cases) not employed in GEP. Overall, in the 46 cases, 23 cases were classified as PCA2 by the GEP/qRT-PCR approach. iv) Clinical/biological correlations. No association was found between the 6-gene signature and IGHV status, SOX11 overexpression, or the presence of the main recurrent mutations (ATM, BIRC3, CCND1, KMTD2, NOTCH1, TP53, TRAF2, WHSC1). Finally, an "ad-interim" analysis of progression free survivals (PFS) suggested a shorter PFS (2-years PFS 76% vs 44%, p=0.04) for PCA2 cases. v) Application of the 6-gene signature to FFPE samples. By testing the signature by qRT-PCR in FFPE samples, 22 and 20 cases were classified PCA1 or PCA2, respectively. Again, PCA2 group demonstrated a trend for shorter PFS (2-years PFS 95% vs 80%, p=0.15). Merging together PB and FFPE cases, PCA2 patients had a shorter PFS (2-years PFS 85% vs 61%, p=0.02). vi) 6-gene signature and sensitivity to the BCR inhibitor ibrutinib. We investigated the proliferation rate of the MCL cell lines Rec-1, Jeko-1, Mino, JVM-2, JVM-13, and Z-138 in presence or in absence of ibrutinib 10 nM for 7 days. By qRT-PCR sensitive cell lines showed higher expression levels of the selected six genes, and were classified as PCA2. A novel 6-gene expression signature related to the BCR pathway characterizes MCL cells with peculiar biological features and sensitivity to BCR inhibitors.

C085

TP53 AND KMT2D MUTATIONS PREDICT PFS IN MCL PATIENTS TREATED WITH HIGH-DOSE THERAPY AND AUTOLOGOUS TRANSPLANTATION: RESULTS FROM THE MCL0208 PHASE III TRIAL FROM FONDAZIONEITALIANA LINFOMI (FIL)

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Recent studies described the landscape of mutated genes in mantle cell lymphoma (MCL). However, little is known about the clinical relevance of these mutations. Thus, we performed deep sequencing of a MCL gene panel in the prospective series of paRecent studies described the landscape of mutated genes in mantle cell lymphoma (MCL). However, little is known about the clinical relevance of these mutations. Thus, we performed deep sequencing of a MCL gene panel in the prospective series of patients enrolled in the FIL-MCL0208 phase III trial (EudraCTNumber: 2009-012807-25, high-dose immunochemotherapy followed by autologous transplantation for untreated, advanced stage <65 years MCL). The mutational study included a subgroup of patients with availability of tumor DNA. A targeted resequencing gene panel, including coding exons and splice sites (ATM, BIRC3, CCND1, KMT2D, TP53, TRAF2, WHSC1, NOTCH1) was analyzed in tumor DNA from baseline bone marrow CD19+ purified MCL cells and, to filter out polymorphisms, in the paired normal genomic DNA (55% of cases) using a TruSeq Custom Amplicon target enrichment system followed by deep next generation sequencing (Illumina, median depth of coverage 2356x). Variants represented in >10% of the alleles were called with VarScan2 with the somatic function when the germline DNA was available. For patients lacking germline DNA, a bioinformatic pipeline with stringent filters was. Primary endpoint was progression free survival (PFS) and clinical data were retrieved at the time of the first interim analysis (May, 2015). Out of the 300 enrolled patients, 174 were evaluable for the mutational study (median age: 57 years; males 75%). The MIPI was intermediate or high-risk in 39%, the Ki67 ≥30% in 44%, and blastoid histology in 9%. Patients not included, due to unavailable tumor DNA (n=124) showed superimposable clinical features, except from a lower stage IV rate (79% vs 91%, p=0.003), as expected. Median follow-up of alive patients was 26 months. At 2-years, 79% of patients were progression free and 91% alive. Overall, at least one mutation was detected in 114/174 cases (66%), including ATM in 43%, WHSC1 in 16%, CCND1 in 13%, KMT2D in 12%, TP53 in 8%, NOTCH1 in 8%, BIRC3 in 5% and TRAF2 in 1%. By univariate analysis, mutations of TP53 (2y PFS 42% vs 83%; p<0.0001) and KMT2D (2-years PFS 69% vs 81%; p=0.008) associated with a shorter PFS. By multivariate analysis, mutations of TP53 (HR: 5.3) and KMT2D (HR: 2) associated with an increase of the hazard of progression. Three MCL subgroups were hierarchically classified (Figure 1). The high-risk category (TP53 +/- KMT2D mutation, 7% of patients) showed a 2y PFS of 42%, the intermediate risk (KMT2D mutation without TP53 mutation, 10%) a 2y PFS of 75%, while the low-risk (no TP53, no KMT2D mutations, 83%) a 2y PFS of 83%. The low number of events so far recorded prevented any analysis on overall survival. Though limited by the short follow-up, our data show that: i) the combination of TP53 and KMT2D mutations predicts a shorter PFS in younger MCL; ii) intensive chemotherapy does not overcome the negative impact of TP53 mutations; and iii) KMT2D mutations may represent a novel genetic biomarker.

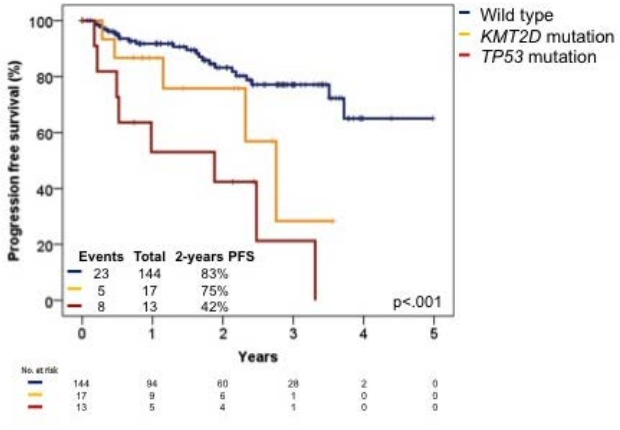

Figure 1.

C086

ION SEMICONDUCTOR SEQUENCING OF CIRCULATING IGH GENE REARRANGEMENTS IN MULTIPLE MYELOMA

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Introduction: Molecular monitoring of B-cell malignancies with PCR-based strategies is currently used to assess the depth of treatment response, detect MRD, and identify patients at increased risk of relapse. IgH gene rearrangements are used as molecular markers in approximately 80% of patients as they represent lineage-specific markers and the CDR3 is unique to each clone. Next-generation sequencing (NGS) provides the opportunity to identify and quantify clonotypes with high sensitivity and universal applicability. Recently, NGS has been applied to characterize the tumor-specific IgH recombination in the plasma of DLBCL patients and used as a non-invasive strategy for clinical disease recurrence after first-line treatment (Roschewski *et al*, 2015). We characterized circulating IgH gene rearrangements in 13 MM patients to assess whether the analysis of cell-free DNA (cfDNA) can be used to overcome biopsy limitations and improve disease monitoring.

Methods: Paired samples of peripheral blood and bone marrow from 13 MM patients were collected. Genomic DNA (gDNA) was extracted from BM CD138+ tumor cells (MachereyNagel) and cfDNA from plasma samples (Qiagen). All DNAs were amplified using seven different family-specific IgHV primers and a consensus JH primer (Voena *et al.*, 1997). NGS libraries were prepared using the Ion Plus Fragment library kit. Barcoded samples were pooled in equimolar concentrations and sequenced using a 316 Ion Chip on a Ion Torrent PGM. Only reads with a median Phred >20, length >250bp and presence of both forward and reverse primers were retained. Filtered reads were aligned using IgBlast against IMGT germline database. Java scripts were used to parse IgBlast output, isolate the CDR3 sequences, aggregate reads into clonotypes and perform frequency-based corrections (MigMap). Post-processing analysis were performed using VDJtools and customized R scripts.

Results: gDNA and cfDNA amplification yielded PCR products of 310-360bp enabling to cover the entire IgH-VDJ region. A custom bioinformatic analysis workflow for quality assessment, alignment and analysis of high-throughput sequencing data was defined. A mean of 60000 and 45000 reads passed quality filters in gDNA samples and cfDNA, respectively. 98% of filtered reads mapped against the germline genes. Both plasma and tumor samples revealed a high level of heterogeneity with a mean of 1400 and 1200 clonotypes respectively and only a subset of these were shared (30±7%). In 11 out of 13 patients, the clonotype with the highest frequency in plasma corresponded to the one identified in the tumor sample. The tumor-associated clonotype could be tracked over time on serial plasma samples showing patient-specific modulation.

Conclusions: cfDNA can be used to identify and monitor the tumor-associated clonotype in MM. In addition, our workflow gives insights into tumor heterogeneity and repertoire complexity, possibly allowing the characterization of clonal expansions and tumor kinetics.

C087

NEXT GENERATION SEQUENCING IN MYELOID MALIGNANCIES AS AN EFFECTIVE STRATEGY TO CHARACTERIZE PATIENTS FOR PERSONALIZED THERAPIES

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Introduction: In the last years,next generation sequencing (NGS) has made its way into the molecular approach in identifying genetic aberrations in different neoplasms. Mutational information is important for biological subclassification,risk stratification and therapeutic decisions. A multigenic NGS panel can be helpful in early detection of small clones,monitoring disease progression and the inclusion in target therapy protocols.

Methods: We performed 15 runs with the Illumina TruSight Myeloid Panel,for a total of 118 patients at diagnosis with myeloid neoplasms:91 AML,15 MPN,3 CML,7 MDS and 2 CMML. 25 patients (21,2%) had a normal karyotype,25 (21,2%) presented one or two alterations,26 (22%) had a complex karyotype,while for 42 patients (35,6%) no information about the karyotype was available. This NGS panel allows to screen somatic variants in 54 genes relevant in myeloid diseases:15 full genes(exons only) and oncogenic hotspots of 39 genes.

Results: The output data were analyzed with the Illumina software Variant Studio and the results were filtered by a coverage of minimum depth of 500 and allele frequency >5%. The mean coverage was 3662 with a mean of 24 alterations per patient. Variants classified as SNP, silent or synonymous were then removed.53.5% of the alterations had a VAF<10%,7.9% between 10-30% and 38.6% >30%. According to Poisson model,the most mutated genes resulted to be ASXL1,ATRX,BCOR,BCORL1,CUX1, DNMT3A,EZH2,KDM6A,KIT,NOTCH1,RUNX1,STAG2,TET2 and TP53 (p<.01). Then, we validated the mutations >10% of NPM1,DNMT3A,TP53,FLT3,JAK2,MPL,SETBP1,IDH1,IDH2,KDM6A,CEBPa and SF3B1 with conventional molecular methods (Sanger Sequencing and dPCR) and all the mutations were confirmed. We also validated the alterations <10% of CEBPa,TP53,RUNX1,IDH1,IDH2,DNMT3A,CALR and FLT3 with the Roche GS Junior 454 and we obtained >98% of concordance. 18 AML samples were also analyzed by WES and 97,5% of the mutations were confirmed. Moreover,multinomial regression was carried out and a predictive model was built to stratify the patients. We found that some clinical status can be predicted by mutated genes as follows: AML(ATRX,BCORL1, CUX1,DNMT3A,KIT,NOTCH1,RUNX1,TET2),MDS(ASXL1,ATRX,BCORL1,CUX1,EZH2,KIT,NOTCH1,RUNX1,TET2,TP5), MPN(CUX1,NOTCH1,TET2,KIT,STAG2,TP53).

Conclusions: These data suggest that a NGS multigenic panel is an effective strategy in myeloid neoplasms characterization and stratification in light of the development of novel personalized therapies. Moreover,this approach is cheaper and time-saving and can reveal alterations with a higher sensitivity than conventional methods. For this reason,this approach could be strongly recommended for all new diagnosis/relapse of myeloid neoplasm in order to obtain a premature complete characterization of the disease that will give advantages in term of therapeutic approach and OS.

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C088

AN INTEGRATED LABORATORY APPROACH FOR THE DIAGNOSIS OF ACUTE PROMYELOCYTIC LEUKEMIA WITH ATYPICAL PML/RARA FUSION TRANSCRIPTS

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Introduction: APL is characterized by the reciprocal translocation t(15;17)(q22;q12) resulting in the fusion gene PML/RARA. Once the diagnostic suspicion of APL has been raised, international guidelines recommend prompt initiation of tailored therapy and proper supportive care in concomitance with the rapid detection of PML/RARA fusion gene. RT-PCR represents the gold standard for genetic confirmation of APL, as it allows the identification of the specific PML/RARA isoform. However, other techniques including conventional karyotype, FISH, and PML nuclear distribution (by immunofluorescence or immunohistochemistry) can be used for diagnostic confirmation. Depending on PML breakpoint, usually located in intron 6, exon 6 or intron 3, different PML/RARA transcript isoforms may be generated, i.e. long (bcr1), variant (bcr2) and short (bcr3) respectively. The long and short isoforms are detected in 95% of APL cases, whereas only 5% harbor the variant form. We report here on the characterization of 3 APL cases harboring atypical PML/RARA transcripts which were not clearly detected after standard RT-PCR amplification.

Methods: RT-PCR and Q-RT-PCR were carried out on diagnostic bone marrow samples (or peripheral blood if bone marrow sample was not available) using standard protocols (van Dongen JJM et al. Leukemia 1999, Gabert J et al. Leukemia 2003, Grimwade D et al. J Clin Oncol 2009). Sanger direct sequencing of PML/RARA was used to better characterize breakpoint junctions (Figure 1). Immunophenotypic analysis was performed by multiparametric flowcytometry. Cytogenetic and FISH studies were carried out according to standard methods.

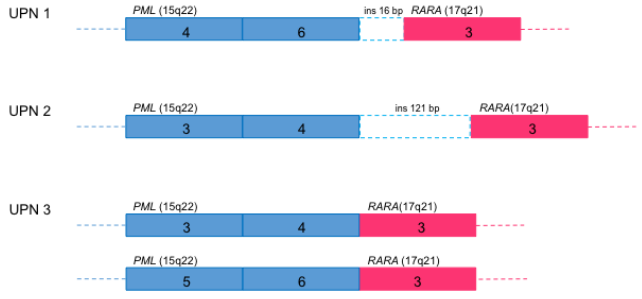

Figure 1. Schematic representation of atypical PML/RARA breakpoints in UPN1, 2, 3.

Results: From 2012 to 2016, we identified 3 patients (UPN 1, 2, 3) showing inconclusive results at RT-PCR routine analysis. In all cases, clinical, morphological and immunophenotypic features were consistent with APL. In addition, 2/3 cases showed a positive cytogenetic analysis for t(15;17). RT-PCR amplification of PML/RARA revealed abnormal size products in the three cases. Q-RT-PCR showed in 2 of 3 cases a low copy number of PML/RARA transcripts. Direct sequencing allowed to identify atypical breakpoints within the PML region (Figure 1). UPN1 showed a deletion of exon 5 and an insertion of 16 bp between exon 6 of PML and exon 3 of RARA. In UPN 2 we identified a novel exon 4 breakpoint in the PML gene and an insertion of 121 bp upstream of RARA exon 3. Finally, UPN 3 showed the simultaneous presence of a classical bcr1 and an additional breakpoint within exon 4 of PML region thereby originating two distinct transcripts.

Conclusions: Despite the rarity of APL cases with atypical PML/RARA fusion, this study indicate that an integrated laboratory approach employing more than one technique is needed to establish a correct APL diagnosis, particularly in cases with clinico-biological features highly consistent with APL. This will allow institution of proper specific treatment with high cure rate potential.

Myelodysplastic and Myeloproliferative Syndromes

C089

TET2 MUTATIONAL STATUS IN LOW GRADE MYELODYSPLASTIC SYNDROME (MDS) PATIENTS AND ASSOCIATED CHANGES IN GENE EXPRESSION

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Background: TET2 is the most frequently mutated gene in MDS (12-26%). We analysed TET2 (ex3-11) mutations in 122 newly diagnosed MDS pts enrolled in an Italian multicentre prospective study (ClinicalTrials.govIDNCT01291745). TET2 mutation was correlated with changes in gene expression (GE) comparing TET-wild type (WT) and Mutated (Mut) pts.

Methods: gDNA from bone marrow was subjected to mutational screening analysis using combined high resolution melting (HRM) analysis and Sanger sequencing (SS). HRM analysis was performed on the 7900HT-RT-PCR (Applied) using primers designed for TET2 isoform A gene locus (NM_001127208). Samples displaying aberrant melting curves were subjected to SS (ABI3130, Applied). All mutations/alterations were first compared with published SNP data (ncbi.nlm.nih.gov/ project/SNP). We analysed mRNA expression in 10 TET2-Mut vs 6 WT samples using Stem Cell (SC) TaqMan Array MicroFluidic Card (Applied Biosystems) and using Expression BeadChip array (Illumina Inc); the intensity files were loaded into the Illumina Genome Studio software for quality control and GE analysis. Quantile normalization algorithm was applied to correct systematic errors: values having a detection score <.05 were excluded and missing values were imputed. Protein levels were determined by Western blotting (WB).

Results: Of the 122 pts, we identified 2 previously described and 16 novel mutations of the TET2 coding sequence: 10 InDel, 4 amino acid substitutions (S820G, M1028I, S1898Y, S1898P) and 2 substitutions coding a STOP codon predicted to alter/abrogate TET2 protein function. Three mutations fell within the highly conserved LCX1 and 2 in LCX2, and were found in pts with a low IPSS. Our incidence of detectable TET2 mutations was 15%. Of the 96 SC genes analyzed from 10 IPSS low-risk pts with TET2-Mut, higher levels of PTEN, FN1, DES, IL6ST, UTF1 with lower levels of IFITM1 mRNA in TET2-Mut compared to TET2-WT cases were detected. GE supervised analysis of microarray data showed four genes ($p<.05$) down-regulated in TET2-Mut vs TET2-WT: ANP32A, HIPK2, HMGB1, and LCOR. WB analysis confirmed higher PTEN levels in 5/7 cases and these 5 cases also showed lack of AKT phosphorylation (pAKT, a protein negatively regulated downstream by PTEN).

Conclusions: We have confirmed the incidence of TET2 mutations in low-risk IPSS pts (15%). Further, we identified an alteration of the PTEN pathway in this subset of MDS pts having TET2-Mut. Here, the increased expression of oncogene PTEN observed in low-risk MDS pts with TET2 mutation could in part be supported by absence of pAKT, its downstream target. Furthermore, GEP identified down-regulation of HMGB1 and IFITM1 expression in TET2-Mut pts, these genes are involved in inducing and sustaining the inflammatory process in the BM environment. TET2 mutated pts with lower HMGB1 levels would have a reduced apoptotic cell load in the BM via "normal" phagocytic mechanisms, important for maintaining clearance of apoptotic cells and BM homeostasis.

C090

A GENOME WIDE ASSOCIATION STUDY BY IMMUNOCHIP REVEALS POTENTIAL MODIFIERS IN MYELODYSPLATIC SYNDROMES

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Introduction: MDS are a heterogeneous group of clonal haematologic diseases, characterized by dysplastic haemopoiesis and by a variable degree of peripheral cytopenia. As clinical and laboratory findings suggest that a marked dysregulation within the immune system may play a central role in the MDS pathogenesis, in the present study we analysed a large cohort of MDS patients from a homogeneous population of Sardinian individuals, by exploiting the so called Immunochip.

Methods: Immunochip is a genotyping array exploring 147,954 single nucleotide polymorphisms (SNPs) localised in genomic regions displaying some degree of association with immune-mediated diseases or pathways. The population studied here includes 4,027 individuals: 133 cases and 3,894 controls from the island of Sardinia, in Italy. Samples were genotyped using the Illumina ImmunoChip array according to the manufacturer's protocol. Altogether 153,978 autosomal markers and 971 non-autosomal markers were genotyped. After applying Bonferroni correction, association results were considered genome-wide significant when p-value was less than $1x10^{-6}$.

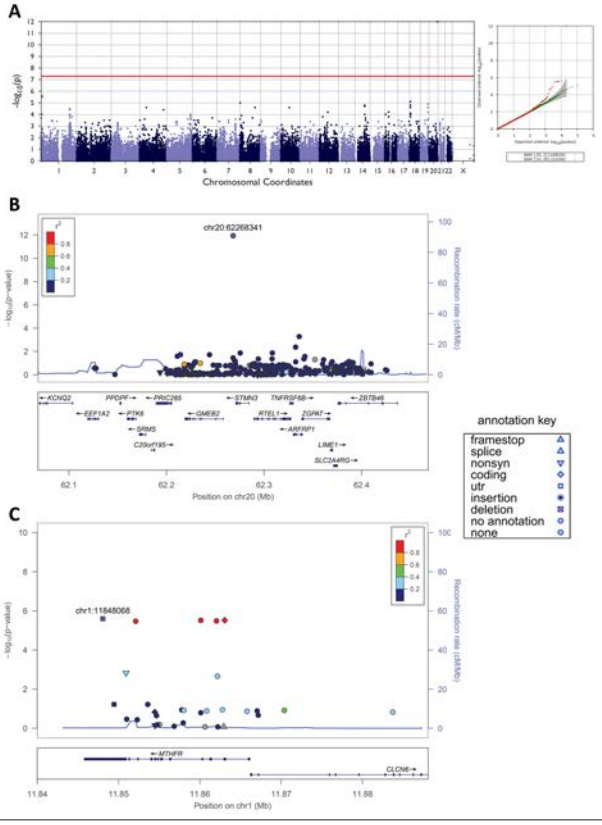

Figure 1.

Results: After association analysis only one variant passed genome-wide significance threshold: rs71325459 ($p=1.16x10^{-12}$), situated on chromosome 20. The variant is in high linkage disequilibrium with rs35640778, an untested missense variant situated in the RTEL1 gene. This gene is an interesting candidate that encodes for an ATP-dependent DNA helicase implicated in telomere-length regulation, DNA repair and maintenance of genomic stability, so that cells from mutated patients show hallmarks of genome instability.

The second most associated signal is composed of five variants that fall slightly below the genome-wide significance threshold but point out another interesting gene candidate. These SNPs, with p-values between $2.53x10^{-6}$ and $3.34x10^{-6}$, are situated in the MTHFR gene. In our cohort, the most associated of these five variants, rs1537514, presents an increased frequency of the derived C allele in cases, with 11.4% versus 4.4% in controls. Methylene tetrahydrofolate reductase (MTHFR) is the rate-limiting enzyme in the methyl cycle. Genetic variation in this gene may influence the susceptibility to a number of different disorders and have been strongly associated with the risk of neoplastic diseases.

Conclusions: The current understanding of the MDS biology, which is essentially based on the hypothesis of the sequential development of multiple subclonal molecular lesions, fits very well with the demonstration of a possible role for both RTEL1 and MTHFR genes polymorphisms. In fact, it is very well known that genetic variations specifically involving these genes are related to a variable risk of genomic instability. Functional studies may further dissect the potential functional link between these two genes and the most relevant molecular defects driving the MDS clonal tides.

C091

EFFECT OF AZACITIDINE AND LENALIDOMIDE THERAPY ON PHOSPHOLIPASE C BETA1 SIGNALLING IN HIGH-RISK MDS

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Introduction. Among the inositide-dependent signalling enzymes, phosphoinositide-specific phospholipase C (PI-PLC) beta1 is implicated in MDS pathogenesis and is epigenetically regulated. Indeed, PI-PLCbeta1 promoter is a specific target for azacitidine, a demethylating agent that is clinically used in MDS to improve patients' overall survival and delay the AML evolution. Moreover, azacitidine is currently used alone or in combination with other drugs, such as growth factors or lenalidomide. As for this latter, the rationale of combining azacitidine and lenalidomide in MDS is linked to the capability of both drugs to balance proliferation and differentiation processes, sustaining both myeloid and erythroid lineages. However, the molecular effect of this combination therapy is still unclear, and the effect on inositide signalling pathways has not been studied yet.

Methods. We studied 44 patients diagnosed with high-risk MDS who were given azacitidine and lenalidomide. Patients were considered clinically evaluable after at least 6 cycles of treatment. Molecular analyses were performed at baseline and during the therapy. At first, Real-Time PCR and immunocytochemical experiments were performed to determine PI-PLCbeta1 expression. Then, we also carried out cell cycle and flow cytometric analyses, and studied both PI-PLCbeta1 methylation status and the expression of erythroid-specific molecules, i.e. Globin genes. Finally, we investigated the effect of the therapy on microRNA expression.

Results. Our study included 44 patients, but only 28 subjects were clinically evaluable, with an overall response rate of 78.6% (22/28

cases). At a molecular level, a significant increase of PI-PLCbeta1 expression was associated with a favourable clinical response to the combination therapy. Moreover, responder cases also showed an increased expression of Beta-globin and erythroid-specific surface markers, such as Glycophorin A and CD71, hinting at a specific contribution of lenalidomide on erythroid activation. On the other hand, the frequent demethylation of PI-PLCbeta1 promoter in responder cases could be specifically linked to azacitidine. Furthermore, MDS cells treated with azacitidine and lenalidomide not only showed an increased G0/G1 phase of cell cycle, but also microRNA expression was affected.

Conclusions. Our results show that the combination of azacitidine and lenalidomide in high-risk MDS patients can be important to induce PI-PLCbeta1. This enzyme, in turn, can regulate cell cycle, myeloid and erythroid differentiation, thus improving peripheral cytopenia.

C092

DYSEGULATED CDC20, P53 AND MITOTIC CHECKPOINTS IN ACUTE MYELOID LEUKEMIA WITH ABNORMAL CHROMOSOME NUMBER DEFINE POTENTIAL THERAPEUTIC TARGETS FOR POOR PROGNOSIS CASES

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Introduction: Chromosome number alteration, aneuploidy, causes unfitness in eukaryotic cells and is generally not sufficient to induce malignant transformation. However, it is a hallmark of cancer and 10-15% of acute myeloid leukemia (AML) cases are aneuploid. The study aimed to investigate the molecular mechanisms activated by aneuploid (A-)AML cells to overcome the unfitness barrier and tolerate an abnormal chromosome number.

Methods: We performed whole exome sequencing of 42 A-AML and 34 euploid (E-)AML (Illumina platform). Variants were called by MuTect and Varscan 2.0. Gene expression profiling was obtained from bone marrow cells of 22 A-AML and 27 E-AML (HTA 2.0, Affymetrix). Data were analyzed using TAC 3.0 (Affymetrix) and GSEA (Broad Institute).

Results: A-AML showed reduced expression of RAD50 compared to E-AML ($p<.001$), suggestive of an impaired DNA damage response and checkpoint arrest. The defect hampered the activation of p53, the guardian of ploidy and its transcriptional program, which were disrupted both at functional and genomic levels. A-AML was not only enriched for TP53 mutations, but also for genomic lesions targeting p53-related genes, including those involved in p53 activation (APAK, FATS, PIAS4) and stability (USP10, DDX31) and known p53 targets (RBM38, DDR1). Overall, 42% A-AML had a mutation in TP53 or its related genes compared with 15% E-AML ($p=.01$). Moreover, a gene expression signature of p53 downregulation was enriched in A-AML, irrespective of TP53 mutational status ($p<.05$). Upregulation of PLK1 ($p<.01$) contributed to functional inactivation of p53 in A-AML. PLK1 also forced entry and progression through mitosis in A-AML by cooperating with overexpression of CDC20 ($p<.001$), which is sufficient to overcome the spindle-assembly checkpoint, leading to aneuploid daughter cells. The spindle assembly machinery and other cell cycle-related genes involved in DNA replication, centrosome dynamics, chromatid cohesion and chromosome segregation were frequently mutated or deregulated in A-AML, suggesting a role in promoting and maintaining genomic instability, which was highlighted by a higher number of genomic alterations in A-AML. The mutations mostly targeted chromatin modifier, splicing, DNA methylation and signaling genes. In parallel, a KRAS transcriptional signature was upregulated in A-AML ($p<.05$), irrespective of the mutational status, to sustain cell survival and proliferation.

Conclusions: We depicted here for the first time the complex molecular mechanisms promoting and maintaining A-AML, which include deregulated checkpoint response, cell cycle machinery, inactivated p53 and hyperactivated KRAS pathway. This evidence suggests that pharmacological reactivation of p53, inhibition of cell cycle checkpoints and KRAS signaling may be valuable therapeutic strategies for aggressive A-AML.

GS,AP: equal contribution

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C093

UPREGULATION OF INDOLEAMINE 2,3-DIOXYGENASE ENZYMES IN LEUKEMIC MESENCHYMAL STROMAL CELLS (MSCS) : A MECHANISM REGULATING MSC/ACUTE MYELOID LEUKEMIA CELL CROSS TALK

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Introduction. Mesenchymal stromal cells (MSCs), an essential element of both normal and leukemic hematopoietic microenvironment, are multipotent cells with a unique immune-modulating ability. Thus, MSCs play a crucial role for both the proliferation and differentiation of hematopoietic stem cells (HSCs) and can induce an immune-tolerant milieu. Indoleamine 2, 3-dioxygenase (IDO1 and IDO2) enzymes catabolize tryptophan to kynurenine and play a key role in the induction of immune tolerance in different settings, including acute myeloid leukemia (AML). Furthermore, IDO1/IDO2 pathway is a well described mechanism by which MSCs exert their immunomodulatory properties. We hypothesized that: 1) MSC-dependent mechanisms are involved in leukemia initiation, maintenance and progression; 2) the expression of IDO1 and IDO2 by MSCs is part of a MSC-dependent mechanism able to create a tumor-supportive milieu.

Methods. We isolated MSCs from the bone marrow of AML patients (AML-MSCs) at diagnosis. We first analyzed their phenotypic and functional properties compared to that of healthy donor-derived MSCs (HD-MSCs). Next, we investigated IDO1/2 expression and functions in in HD-/AML-MSCs and AML cells before and after co-cultures.

Results. We efficiently isolated and expanded AML-MSCs which express typical MSC markers. We found that the frequency of rescued MSCs was lower in AML group than in HD, suggesting a reduced number of MSC precursors in leukemic bone marrow. Moreover, AML-MSCs show a reduced proliferative capacity reflecting an intrinsic defect. However, AML-MSCs analyzed by FISH do not show AML cell cytogenetic abnormalities suggesting that AML-MSCs do not derive from the original malignant clone. Next, we demonstrated that IDO enzymes are expressed in AML-MSCs as well as in HD-MSCs. IDO1 is efficiently upregulated by different inflammatory stimuli, and IDO1 protein expression parallels mRNA in both HD-MSCs and AML-MSCs. Interestingly, IDO2 mRNA is upregulated, after IFN-gamma stimulation, in particular in AML-MSCs, although the level of induction varies between different patients. IDO enzymes expressed by MSCs are functional. Indeed, lymphocyte proliferation is inhibited in a IDO-dependent manner by HD-/AML-MSCs. Interestingly, IDO1/IDO2

expression is upregulated in HD-/AML-MSCs as well as in AML cells after co-coltures in the absence of exogenous inflammatory stimuli. Finally, HD-/AML-MSCs stimulate AML cell proliferation and this effect is, at least in part, IDO-mediated. Indeed, IDO-inhibitor addition reduce AML cell proliferation supported by MSCs.

Conclusions. These findings may help to discover novel niche-target prognostic/therapeutic factors and to provide novel applications for drugs already under active clinical investigation (i.e. IDO-inhibitors).

C094

DIAGNOSTIC VALUE OF PAS POSITIVITY OF BONE MARROW ERYTHROBLASTS IN MYELODYSPLASTIC SYNDROMES

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Introduction. The revised 2008 WHO classification confirmed minimal morphological criteria of myelodysplastic syndrome (MDS) diagnosis: at least 10% of bone marrow (BM) cells of at least one hematopoietic cell lineage must show unequivocal dysplasia to be considered as dysplastic. Morphological abnormalities of erythroid cells include cytoplasmic Periodic acid-Schiff (PAS) positivity, but the PAS positivity diagnostic power is not yet fully clear. Since dysplastic alterations of BM precursors and of peripheral blood cells are still fundamental for diagnostic classifications, the aims of our study were to evaluate the diagnostic value of erythroblast PAS positivity in MDS and to investigate a possible correlation between PAS positivity levels and other morphological and clinical features.

Methods. We retrospectively examined the results of the cytochemical PAS staining for glycogen in BM smears from 151 MDS patients, 117 patients with non-clonal cytopenia and 49 healthy subjects. By counting 100 nucleated cells for the erythroid lineage and classifying them for their degree of PAS reactivity, we developed a PAS score for MDS identification.

Results. PAS positive erythroblasts were observed in 93 (62%) MDS patients, 48 (41%) patients with non-clonal cytopenia and 12 (24%) healthy subjects ($p<0.0001$). In MDS, both positivity rates (mean 3.9%, range 0-31%) and scores (mean 5.8, range 0-49) were significantly higher than those in normal and pathologic controls ($p=0.0001$ and $p=0.0006$, respectively). In MDS, no significant relationship was detected between erythroblast PAS positivity rate or score and dyserythropoiesis grading, multilineage dysplasia or excess blasts, whereas there was an inverse correlation between PAS score values and percentages of BM erythroblasts ($p=0.041$) or percentages of ring sideroblasts ($p=0.012$). Anemic patients showed higher score values than non-anemic subjects ($p=0.016$). PAS positivity was unrelated to karyotype abnormalities, whereas a significant inverse correlation was found between PAS positivity and SF3B1 mutations. A ROC curve analysis allowed us to identify a PAS score value >4 (AUC=0.697, $p=0.0006$) and a PAS positive erythroblast percentage >1 (AUC=0.674, $p=0.0034$) as optimal cut-off to discriminate MDS patients without excess blasts or ring sideroblasts from non-clonal cytopenia. Considering the most discriminant morphological features for dyserythropoiesis, the weight of both PAS positivity rate and score in the recognition of BM dysplasia was lower than that of ring sideroblasts and megaloblastosis, but higher than that of defective hemoglobinisation, nuclear lobulation, multinuclearity, cytoplasmic fraying, pyknosis, and internuclear bridges.

Conclusions. The evaluation of BM erythroblast PAS positivity may be useful in the work-up of patients with suspected MDS. This parameter should be included in the diagnostic morphological panel to be used for a correct application of the WHO classification.

C095

MIR-494-3P OVEREXPRESSION SUPPORTS MEGAKARYOCYTOPOIESIS IN PRIMARY MYELOFIBROSIS CD34+ HEMATOPOIETIC PROGENITOR CELLS THROUGH THE DOWN-REGULATION OF SOCS6

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Introduction: Primary Myelofibrosis (PMF) belongs to BCR-ABL negative Myeloproliferative Neoplasms (MPNs) and is characterized by hematopoietic stem-cell derived clonal myeloproliferation, involving especially megakaryocyte (MK) lineage, bone marrow fibrosis and extramedullary hematopoiesis. Recent studies have suggested that miRNA could play a critical role in MPN's pathogenesis. According to this, we previously performed the integrative analysis of gene and miRNA expression profiles of PMF CD34+ hematopoietic progenitor cells (HPC) and identified miR-494-3p as the upregulated miRNA associated to the highest number of anti-correlated predicted targets.

Methods: In this study we performed miR-494-3p overexpression experiments in cord blood (CB) CD34+ cells and evaluated its effects on HPC differentiation through immunophenotypic and morphological analysis, and by clonogenic assays. We also performed inhibition experiments in PMF CD34+ cells by means of Locked Nucleic Acid antisense oligonucleotides targeting miR-494-3p. Next, we studied the Gene Expression Profile (GEP) of CB CD34+ cells overexpressing this miRNA using microarray. We confirmed GEP results by means of Real-Time quantitative Reverse Transcription PCR (qRT-PCR) and western blot analysis. Furthermore, we set up 3' UTR Luciferase Reporter Assay to study the interaction between miR-494-3p and its predicted target Suppressor of Cytokine Signaling 6 (SOCS6). Finally, we performed SOCS6 silencing experiments in CB CD34+ cells and the effects on HPC differentiation were evaluated through morphological and immunophenotypic analysis, and by clonogenic assays.

Results: miR-494-3p overexpression promoted MK differentiation in CB CD34+ cells as demonstrated by flow cytometric analysis, collagen based clonogenic assay and morphological analysis. Conversely, miR-494-3p inhibition impaired megakaryocytopoiesis in PMF CD34+ cells. GEP of CD34+ cells overexpressing miR-494-3p allowed the identification of 20 genes downregulated both after miRNA overexpression and in PMF CD34+ cells. Among them, we identified SOCS6 as the miR-494-3p predicted target associated to the most favourable context+ score according to TargetScanHuman. Real-Time qRT-PCR and western blot results confirmed the downregulation of SOCS6 after miR-494-3p overexpression. Furthermore, 3' UTR Luciferase Reporter Assay proved that SOCS6 is a real miR-494-3p target. SOCS6 silencing experiments revealed that its downregulation supported megakaryocytopoiesis in CB CD34+ cell.

Conclusions: Our results demonstrate that miR-494-3p overexpression, observed in PMF CD34+ cells, promotes megakaryocytopoiesis in HPC. Moreover, we proved that SOCS6 is a direct target of miR-494-3p and that its downregulation exert the same effect of miR-494-3p overexpression on HPC differentiation. Altogether,

these results suggest that miR-494-3p/SOCS6 axis could be involved in the induction of MK hyperplasia typically observed in PMF patients.

C096

THE INFLAMMATORY MICROENVIRONMENT INDUCES A MUTATION-ASSOCIATED FUNCTIONAL DYSREGULATION OF THE CIRCULATING MEGAKARYOCYTE PROGENITORS OF MYELOFIBROSIS

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Introduction: Myelofibrosis (MF), a clonal disorder of the hemopoietic stem/progenitor cells, is characterized by distinctive abnormalities in megakaryocyte (MK) development. In addition to driver molecular mutations in Janus Kinase 2 (JAK2) and Calreticulin (CALR) genes, chronic inflammation has emerged as a key-player in MF pathogenesis. Here, we analyzed the potential contribution of crucial factors of the inflammatory microenvironment (Interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α and Tissue Inhibitor of Metalloproteinases (TIMP)-1 to MF dysmegakaryocytopoiesis.

Methods: To characterize the mutational status, JAK2V617F and CALR exon 9 mutations were assessed by quantitative PCR–based allelic discrimination assay and Next Generation Sequencing approach, respectively. To quantify MK progenitors, circulating CD34+CD41+ cells of MF patients (10 JAK2V617F and 6 CALR mutated) and controls (cord blood (CB); 8 cases) were identified by flow cytometry analysis. To evaluate the pro-inflammatory profile, IL-1β, TNF-α and TIMP-1 were measured in plasma of patients (10 JAK2V617F and 6 CALR mutated) and healthy subjects (10 cases) by ELISA. To determine the in vitro effect of inflammatory factors on megakaryocytopoiesis, Colony Forming Units-Megakaryocyte (CFU-MK) growth of circulating CD34+ cells from MF patients (4 JAK2V617F and 4 CALR mutated) and CB (4 cases) was assessed with a collagen-based medium in the presence or absence of TIMP-1, TNF-α and IL-1β, alone or in combination. Pure and mixed CFU-MK were quantified on the basis of CD41 immunostaining.

Results: We found that the frequency of circulating MF-derived CD34+CD41+ was significantly higher than the CB counterparts. However, a significantly higher proportion of cells was associated with CALR compared to JAK2V617F mutated patients. Irrespective of mutational status, IL-1β, TNF-α and TIMP-1 plasma levels were significantly increased in MF patients. In addition, we demonstrated that the growth of pure CFU-MK from untreated cells of JAK2V617F mutated patients was significantly reduced as compared to the CALR mutated and the CB counterparts. When inflammatory factors alone were tested, we found that, at variance with CB, the growth of MK progenitors from MF patients is significantly inhibited by TNF-α, irrespective of mutation status. In contrast, IL-1β has stimulatory activity on pure MK colony formation of JAK2V617F, but not CALR, mutated patients. TIMP-1 was ineffective. Factors in combination did not significantly modify the growth of patients/CB pure CFU-MK as compared with factors alone. At variance with JAK2V617F mutated patients, TNF-α (alone or in combination), but not IL-1β, increases the growth of mixed MK colonies of CB and of patients harboring CALR mutation.

Conclusions: Here we demonstrated that signals provided by the inflammatory microenvironment induce a mutation-associated functional dysregulation of the circulating MK progenitors of MF.

POSTERS

Acute Leukemia 1

P001

CHARACTERIZATION OF PATIENTS WITH RELAPSED OR REFRACTORY AML IN CONTINUED FOLLOW-UP AFTER TREATMENT WITH VOSAROXIN/CYTARABINE VS PLACEBO/CYTARABINE IN THE VALOR TRIAL

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Background: Patients with relapsed/refractory (R/R) AML have a median overall survival (OS) less than 1 year. In the phase 3 VALOR trial, vosaroxin/cytarabine prolonged median OS in patients with R/R AML by 1.4 months vs placebo/cytarabine (7.5 vs 6.1 months; HR = 0.87 [95% CI 0.73-1.02]; P = 0.061). Of 711 enrolled patients, 134 (19%) were alive in follow-up at the primary analysis. Here, we characterize VALOR patients in continued follow-up.

Methods: In VALOR, patients with R/R AML were randomized 1:1 to receive cytarabine (1 g/m^2 IV over 2 h, d 1-5) plus either vosaroxin (90 mg/m^2 IV over 10 min d 1, 4; 70 mg/m^2 in subsequent cycles) or placebo. After the primary analysis, ongoing patients were followed for survival.

Table 1. Baseline Characteristics of Ongoing Patients.

	Vos/Cyt (n=46)	Pla/Cyt (n=37)
Median (range) age, y	59.5 (23-74)	54 (23-69)
< 60 y, n (%)	23 (50)	27 (73)
≥ 60 y, n (%)	23 (50)	10 (27)
Disease status, n (%)		
Refractory	10 (22)	11 (30)
Early relapsed	18 (39)	10 (27)
Late relapsed	18 (39)	16 (43)
Geographic location, n (%)		
US	15 (33)	16 (43)
Ex-US	31 (67)	21 (57)
ECOG performance status, n (%)		
0	32 (70)	27 (73)
1	11 (24)	9 (24)
2	3 (7)	1 (3)
Cytogenetics by NCCN Guidelines, n (%)		
Favorable	3 (10)	3 (11)
Intermediate	23 (79)	19 (68)
Unfavorable	3 (10)	6 (21)
Missing	17	9

ECOG, Eastern Cooperative Oncology Group; NCCN, National Comprehensive Cancer Network.

Results: As of Jan 22, 2016, 83 patients (12%) were alive in follow-up: 46/356 (13%) in the vosaroxin/cytarabine arm and 37/355 (10%) in the placebo/cytarabine arm. Median follow-up in these patients was 40 months (range 28-60). Patient characteristics are presented (Table 1); a higher proportion of patients were ≥ 60 years in the vosaroxin/cytarabine arm (50% vs 27% with placebo/cytarabine). Most achieved complete remission (CR) on study (70% with vosaroxin/cytarabine; 51% with placebo/cytarabine); over half maintained CR at database lock (59% with vosaroxin/cytarabine; 49% with placebo/cytarabine). Nearly all received subsequent therapy (93% with vosaroxin/cytarabine; 100% with placebo/cytarabine). Most patients on vosaroxin/cytarabine (85%) and all patients on placebo/cytarabine received posttreatment stem cell transplantation (SCT). Seven patients in the vosaroxin/cytarabine arm did not undergo SCT; all were ≥60 years of age. Median follow-up in these 7 patients was 33 months (range 31-48).

Conclusions: A small proportion of patients with R/R AML continue to be followed for survival in VALOR. Typically, these patients achieved CR followed by SCT; however, some patients ≥ 60 years treated with vosaroxin/cytarabine achieved long-term survival without SCT.

P002

INCIDENCE AND CLINICAL SIGNIFICANCE OF CENTRAL NERVOUS SYSTEM (CNS) INVOLVEMENT IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) DETERMINED BY CITOMORPHOLOGIC AND FLOW CYTOMETRY IN CEREBRAL SPINAL FLUID

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Introduction: In Acute Lymphoblastic Leukemia (ALL), patients (pts) with Central Nervous System (CNS) involvement at diagnosis have an increased risk of relapse and require intensified CNS targeted therapy. Since conventional cytology (CC) of cerebrospinal fluid (CSF) may fail to demonstrate malignant cells, especially in samples with low cell counts, flow cytometry (FCM) may help discriminate further conditions of leukemic infiltration.

Methods: The aims of our study were to: 1) determine the incidence of manifest or occult CNS disease in a series of adult pts with ALL; 2) correlate CNS disease with the clinico-biologic parameters; 3) examine the impact of CNS involvement on outcome. We collected CSF samples from 84 pts with a newly diagnosed ALL, 43 females and 41 males, median age 42 years (range 17-75), median white blood cells count (WBCc) 10.6x10^9/L (range 1-374x10^9/L). Sixty-tree pts (75%) had a B lineage-ALL. The cytogenetic/genetic information was available in 46/84 (55%) pts: 16 (19%) and 5 (6%), had BCR/ABL and MLL rearrangement, respectively. Pts were treated according to the GIMEMA and NILG ALL protocols or with Hyper-CVAD program. Thirty-five pts underwent allogeneic stem cell transplant (ASCT). All CSF samples were examined by CC and FCM. The presence of ≥10 clonally restricted or phenotypically abnormal events was regarded as FCM positivity.

Results: Overall, 21 (25%) pts were CNS positive (CNS+); 5(6%) were CC and FCM positive (manifest CNS+) and 16 (19%) were only FCM positive (occult CNS+). Median age, median WBCc and cytogenetic/genetic features did not differ significantly between CNS+ and negative (CNS) pts. Of CNS+ pts, 1 (5%) died during

the induction course (ID) and 18 (86%) achieved a complete remission (CR), similar to the CR (87%) and ID (3%) rates of the entire cohort. Of 18 CNS+ pts who achieved CR, 14 experienced hematologic relapse (p=0.03). Overall survival (OS) of CNS+ pts was significantly shorter than CNS- (0% vs 84% at 2 years, p=0.001). In the subset analysis, 1-year OS of manifest CNS+, occult CNS+ and CNS- pts was 0%, 33% and 69%, respectively (p=0.008). Furthermore, CNS involvement identified pts with a significantly shorter OS also among those who received ASCT (0% vs 59% at 1,7 year, p= 0.01).

Conclusions: We conclude that: 1) in adult pts with ALL, FCM allows occult CNS disease to be detected, even under conditions of low leukemic burden; 2) the presence of occult CNS disease anticipate an adverse outcome. Further prospective studies on larger series are needed to confirm this data.

P003

HIGH EXPRESSION OF CD200 IS ASSOCIATED WITH INFERIOR SURVIVAL IN PATIENTS WITH CYTOGENETICALLY NORMAL ACUTE MYELOID LEUKEMIA

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Introduction: Overexpression of CD200, a trans-membrane protein belonging to the immunoglobulin superfamily, has been associated with poor prognosis in patients with acute myeloid leukemia (AML). As few data are available in the subset of cytogenetically-normal (CN) AML, we retrospectively evaluated the correlations between CD200 expression and response to therapy in a series of 122 adults with CN-AML.

Methods: One hundred twenty-two patients with CN-AML, median age 59 years (range: 22-80), treated at our Institutions between 2005 and 2015 were included in this analysis. Blast cells immunophenotype and CD200 expression were evaluated by multi-parametric flow cytometry; high intensity of expression was defined by a MFI >11. All patients received induction chemotherapy (generally based on fludarabine) and, if feasible, at least one consolidation course with high-dose cytarabine.

Results: CD200 was expressed in 54/122 (44%) cases; 15 of them (28% of positive) expressed CD200 at high intensity. No differences in CD200 expression rate were observed according to age, WBC count, type of leukemia, FLT3 or NMP1 mutation, and CD56 expression. Conversely, a higher incidence of CD200 expression was observed in CD34+ cases (34/44 vs 23/81, P<0.0001) and in bcl2+ patients (29/53 vs 25/68, P=0.05). Complete remission (CR) was evaluable in 112 patients: 50/61 (82%) CD200- and 32/51 (63%) CD200+ patients (P=0.04) achieved CR, with a trend towards a lower CR rate in patients with high CD200 intensity (7/14, 50%). CD200 expression had a negative impact on long-term outcome. CD200 expression, per se, did not impact on disease-free survival (DFS), but cases with high CD200 expression had a lower 3-year DFS compared to CD200-negative and low-expressing ones (66% vs 0%, P=0.02). Three-year overall survival was 49% (95%CI: 36-63) in CD200- patients and 30% (95%CI: 17-43) in CD200+ ones (P=0.04), with a significant difference among cases with low or high CD200 expression: 36% (95%CI: 20-53) and 9% (95%CI: 0-25), respectively (P=0.002 in the three groups). Besides CD200 and its intensity, other factors negatively associated with OS in multivariable analysis were bcl2 expression and advanced age (>55 years).

Conclusions: Our data suggest a negative impact of CD200 expression in AML patients, in term of a lower probability of CR and shorter OS. Higher expression of CD200 seems to be associated with a further worsening of long-term outcome, thus identifying a subset of CN-AML patients who could deserve a more intensive therapy.

P004

ROLE OF GSK-3 SIGNALLING IN NON-PROMYELOCITIC ACUTE MYELOID LEUKEMIA(AML) CELL RESPONSE TO CHEMOTHERAPY

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Background and aims: GSK-3 is a serine-threonine kinase involved in metabolic regulation as well as in the control of many pathways associated to cancer development, including Notch Wnt/β-catenin, Hedgehog, and AKT. Association of GSK-3 inhibitors with All-trans-retinoic acid (ATRA) significantly improved ATRA-mediated differentiation and cell death of acute promyelocytic leukemia cells. However, little is currently known about the contribution of GSK-3 role to non-promyelocytic AML cell response to treatment with chemoterapeutic agents.

Methods: GSK-3 expression was analyzed by Western blot or flow cytometry inAML cell lines (HL-60, THP1, U937) or primary non-promyelocytic AML blast cells (30 samples). AML cellscultured alone or in presence ofhuman bone marrow mesenchymal stromal cells (hBM-MSCs)were treated with GSK-3 inhibitors, including LiCL, AR-A014418, SB 216763, in association or not with Cytarabine (Ara-C) or Idarubicin. Cell proliferation and cell death were measured by CFSE dilution and TOPRO-3/Annexin-V staining, respectively.

Results: Flow cytometry and Western blot analysis in AML samples revealed highexpression levels of all GSK-3 forms, including total GSK-3α, (Ser21) GSK-3α, total GSK-3β, and (Ser 21) GSK-3β; theseforms were all down-modulated when AML cells were cultured in presence of hBM-MSCs, thus suggesting that GSK-3 plays an important role intransducting microenvironmental signals in AML cells interacting with bone marrow stroma. The treatment of AML cells with increasing concentrations of each GSK-3 inhibitors decreased AML cell viability in a dose-dependent manner; interestingly,hBM-MSCsor peripheral blood mononuclear cells were less sensitive to GSK-3inhibitors. The addition of each inhibitor increased dramatically the AML cell apoptotic rate induced by the addition of Ara-C or Idarubicin*in vitro*. Notably, LiCl and AR-A014418 were capable of abrogatinghBM-MSC-mediated AML cell resistance to apoptosis induced by Ara-C or Idarubicin.

Conclusion: Our data clearly demonstrate that GSK-3 inhibition may improve the efficacy of some chemoterapeutic agents used in the treatment of non-promyelocytic AML.

P005

THE TARGETABLE ROLE OF HAUSP IN P190 BCR-ABL LEUKEMIA

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Philadelphia-positive Acute Lymphoblastic Leukemia (ALL) is driven by the p190-BCR-ABL isoform. Although effectively targeted by BCR-ABL tyrosine kinase inhibitors (TKI), ALL is associated with a less substantial response to TKI when compared to Chronic Myeloid Leukemia. Therefore, the identification of additional genes required for ALL maintenance may yields essential therapeutic implications to eradicate this cancer. We demonstrate

that p190-BCR-ABL is able to interact with HAUSP and to promote its phosphorylation. Notably, expression of HAUSP-Y243F/Y878F/Y947F, HAUSP-TM (triple mutant), sensibly reduces HAUSP phosphorylation levels. Similarly, treatment with tyrosine kinase inhibitor Imatinib is associated with the dephosphorylation of HAUSP. Beside the ability of HAUSP to de-ubiquitinate PTEN and the relevance of BCR-ABL/HAUSP/PTEN network in CML, HAUSP is also able to target p53. In particular, HAUSP interferes with the MDM2/p53 network causing p53 destabilization. Deletion of HAUSP and inhibition of HAUSP were indeed associated with p53 up-regulation. Our data did not demonstrate whether p53 upregulation is directly regulated by the HAUSP activity or depends on the more complex BCR/ABL-HAUSP-MDM2-p53 network, however the relevant p53 up-regulation arbors such important therapeutic implications that HAUSP inhibitors should be assessed in the clinical setting.

P006

ISOLATION AND CHARACTERIZATION OF MESENCHYMAL STROMAL CELLS DERIVED FROM PAEDIATRIC PATIENTS WITH B ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction: Several studies reported the importance of tumor microenvironment in the development and progression of hematological disorders. The identification of key factors involved in the crosstalk between the malignant cells and the bone marrow Mesenchymal Stromal Cells (BM-MSCs) may provide a tool for interfering with the protective BM niche. The purpose of our work was to isolate and characterize morphologically, phenotypically and functionally MSCs derived from pediatric patients with B Acute Lymphoblastic Leukemia (B-ALL).

Methods: MSCs were isolated from BM-MNCs obtained from 10 B-ALL children (n=5 high risk patients and n=5 t(12;21) patients) and from 6 healthy donors (HDs) and cultured in DMEM 10% FCS. MSCs were characterized at fourth passage in terms of morphology, immunophenotype (FACS analysis) and *in vitro* adipogenic and osteogenic differentiation potential. Chromosomal translocations detected in leukemia cells were investigated in B-ALL-MSCs by fluorescence in situ hybridizations (FISH) or polymerase chain reaction (PCR).

Results: Both HD-MSCs and B-ALL-MSCs resulted comparable in terms of morphology. They both expressed the typical MSC markers CD73, CD90 and CD105, while lacked the expression of the hematopoietic markers CD14, CD34, CD45 and MHC-II. HD-MSCs as well as B-ALL-MSCs were able to differentiate, under appropriate stimuli, into adipogenic and osteogenic lineages as showed by Oil Red O liphophilic dye and Alizarin Red staining of calcium deposits. In addition, MSC from all investigated ALL patients did not present the chromosomal translocations that had been detected in leukemia cells (1 patient BCR-ABL p210, 5 patients TEL-AML1).

Conclusions: We found that B-ALL-MSCs resulted similar in terms of morphology, phenotype and differentiation ability to HD-MSCs. Furthermore, MSCs from patients did not reveal the chromosomal translocations present in leukemia blasts. Functional characterization of MSCs in terms of soluble molecule production is needed to identify altered cellular pathways. Since emerging evidence supports the importance of the MSCs in the leukemic niche, we will focus on potential functional alterations of ALL-MSCs. Our purpose is to understand the mechanisms underlying the support of leukemic cells by the BM microenvironment. The discovery of altered molecular pathways will pave the way for the development of new immunotherapy strategies for targeting the leukemic niche.

P007

A NOVEL SYNTHETIC LETHAL APPROACH TARGETING SIRT6 IN ACUTE MYELOID LEUKEMIA

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Currently available therapeutics against Acute Myeloid Leukemia (AML) have improved patient outcome. However, resistance develops even to novel therapies and five-year patient overall survival remains low, especially for patients who are not eligible for allogeneic bone marrow transplantation. Therefore, there is an urgent need to overcome the biologic mechanisms underlying drug resistance in AML, to enhance the efficacy of existing treatments and to facilitate the design of novel approaches. Recently, our group has demonstrated that SIRT6, a NAD+-dependent histone deacetylase involved in genome maintenance, is frequently up-regulated in Multiple Myeloma and its targeting induces cancer cell killing (Cea M. et al, Blood 2016). Furthermore, gene expression analyses performed by our groups show that SIRT6 is also up-regulated in AML and confers poor prognosis. Here we investigated the role of this NAD+-dependent deacetylase in regulating ongoing DNA damage observed in AML.

Patients were grouped into lower and higher SIRT6 expressers according to its median mRNA level. Importantly, subjects with lower expression had a higher incidence of FLT3-ITD (p=0.034, Wilcoxon signed rank test). No significant association was observed with respect to mutations of NPM1, nor with WT1 and BAALC expression. SIRT6 expression correlated also with adverse clinical outcome in term of event free and overall survival (p=0.035 and p=0.025, respectively; unpaired t test). Based on these data, we evaluated SIRT6 role in biology of AML. We found higher SIRT6 protein level in AML cell lines carrying FLT3-ITD mutation (MOLM-14 and MV 4-11) compared to cell lines harboring other mutations (OCI-AML3, THP-1, KG, NB4, HL60, Nomo1 and U937). Targeting SIRT6 by specific shRNAs weakly reduced AML cell survival compared with control-scrambled cells, by impairing DNA repair efficiency. Indeed, a restricted effect of SIRT6 impairment on DNA damage proteins (H2AX, RAD51, 53BP1, RPA32) was measured. We next examined the therapeutic relevance of SIRT6 inhibition in AML by testing effects of its depletion in combination with genotoxic agents. Remarkably, SIRT6 depletion conferred increased sensitivity of AML cells to idarubicin, Ara-C and Fludarabine. Overall, enhancing genotoxic stress while concomitantly blocking DNA double-strand breaks (DSBs) repair response, may represents an innovative strategy to increase chemosensitivity of AML cells. Further mechanistic studies revealed that SIRT6 acts as a genome guardian in leukemia cells by binding DNA damage sites and activating DNA-PKcs and CtIP by deacetylation, which in turn promotes DNA repair.

Conclusions: Genomic instability is present in all hematologic malignancies including AML. Strategies aimed to shift the balance towards high DNA damage and reduced DNA repair by SIRT6 inhibition can decrease AML growth and may benefit patients with otherwise unfavorable outcomes.

P008

THE INHIBITION OF CHK1 OVERRIDES THE G2/M ARREST INDUCED BY DOXORUBICIN AND PUSHES LEUKEMIC CELLS TO DEATH

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Nowadays several Checkpoint kinase inhibitors (Chk-i) have been developed to specifically inhibit the mechanism of response to DNA damages and consequently increase the toxicity of different genotoxic agents. Our group has already showed the efficacy of this class of compounds in single agent for the treatment of acute lymphoblastic leukemia (ALL). In the present study we evaluated the efficacy of the combination between a Chk1/Chk2 inhibitor (Chk-i) and the topo-isomerase II inhibitor, doxorubicin, for the treatment of acute lymphoblastic leukemia (ALL). The efficacy in single agent of doxorubicin was evaluated on human B (NALM-6, NALM-19 and REH) and T (MOLT-4, RPMI-8402 and CEM) ALL cell lines in term of reduction of the cell viability, modification of cell cycle profile and activation of the DDR pathways. Cells were treated with doxorubicin (0.25-2.5uM) for 24 and 48 hours and the reduction of the cell viability was quantified using WST-1 reagents. In all the cell lines treated the cytotoxic effect of doxorubicin was time and dose dependent. Then we evaluated the effect of the treatment on cell cycle profile (Pi staining) on different ALL cell lines. Due to the inhibitory effect of the compound on the topoisomerase II and due to the activation of the G2/M checkpoint cells were arrested in G2/M phase. Then the effectiveness of the Chk-i as a chemo-sensitizer agent was evaluated on different cell lines. To this purpose cells were treated with doxorubicin (5, 10, 25 and 50 nM for the more sensitive cell lines; 50, 100, 250 and 500 nM for the less sensitive cell lines) in combination with the Chk-i (2, 5 and 10 nM) for 24 and 48 hours. The combination showed a synergistic effect in term of reduction of the cell viability and induction of apoptosis. The effect of the combination was also analyzed using Western blot looking for specific marker of activation of the DDR pathway showing the same synergistic effect. Moreover the effect of the combination on cell cycle profile was evaluated using a double staining Pi/Anti-phospho-Histone H3 ser10 (marker of mitosis). Cell lines were pre-treated for 18 hours with doxorubicin and then with the Chk-i for different time points (1, 2, 3, 6 and 9 hours). The treatment with the Chk-i removed the G2/M arrest induced by the pre-treatment with doxorubicin, progressively reducing the number of cells in G2/M phase, increasing the percentage of cells positive for the mitotic marker p-HH3 (ser10) and increasing the percentage of cells in sub-G1 phase. In our opinion the combination between the Chk-i and the topoisomerase II inhibitor, doxorubicin, could be a promising strategy for the treatment of B/T-ALL.

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P009

THE INHIBITION OF THE DNA DAMAGE RESPONSE (DDR) USING THE WEE-1 INHIBITOR, MK-1775, SENSITIZES ACUTE LYMPHOBLASTIC LEUKEMIA CELLS TO THE TOXICITY OF DIFFERENT COMPOUNDS

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Different kinases are involved in cell cycle regulation. The Wee-1, ATR/Chk1 and ATM/Chk2 pathways are involved in cell cycle regulation, DNA damages response and DNA repair. Wee1 is a checkpoint kinase, involved mainly in the regulation of G2/M transition through the phosphorylation of both Cyclin-dependent kinase 1 (CDK1) and 2 (CDK2). In this study we evaluated the efficacy of a selective Wee-1 inhibitor, MK-1775, in single agent and in combination on acute lymphoblastic leukemia (ALL) cell lines and primary samples. The inhibition of Wee-1 deeply reduced the cell viability in a dose and time-dependent manner in all the treated cell lines. Moreover the reduction of both cell viability and proliferation were associated with the increment of apoptosis and the activation of different DNA damage markers (ÔÅßH2AX and Parp-1), confirming the toxicity of MK-1775 in single agent. Light microscopy analysis showed that the treatment with MK-1775 induced aberrant nuclear structure (micro-nuclei and DNA bridges) highlighting the mechanisms of cell death through the mitotic catastrophe. We hypothesized that targeting Chk1, a kinase upstream, of Wee1, would be more effective in reducing cell proliferation. Indeed, the concomitant inhibition of Chk1 and Wee1 kinases, using the PF-0477736 in combination with MK-1775, synergized in the reduction of the cell viability, inhibition of the proliferation index and induction of apoptosis. We performed further studies to understand the chemo-sensitizer activity of the compound, thus MK-1775 was combined with different drugs (Clofarabine, Bosutinib Authentic, Bos, and a particular isomer of Bosutinib, Bos-I). The combination between MK-1775 and clofarabine showed a synergistic effect in terms of reduction of the cell viability, inhibition of proliferation and induction of apoptosis on both primary leukemic cells and leukemia cell lines. Then the Wee1 inhibitor was combined with the tyrosine kinase inhibitors Bos and Bos-I. Both isomers in combination with MK-1775 showed an additive effect in term of reduction of the cell viability. The cytotoxic effect of Bos-I was stronger on the Philadelphia-negative cell lines in comparison to the positive counterpart. Western blot analysis highlighted that this compound, but not the Bos, interfered with the Chk1/Chk2 and Wee1 pathway. In our opinion the results of this study identify the Wee1 kinase as a promising target for the treatment of ALL. As mono-therapy the inhibition of Wee1 increases the genetic instability of leukemic cells, promoting cell death for the progressive accumulation of DNA damages. As chemo-sensitazer agent, the MK-1775 inhibits the DNA damage response pathway, increasing the cytotoxicity of different compounds

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P010

MULTI-CENTER OBSERVATIONAL STUDY OF PREVALENCE, IMPACT ON SURVIVAL OF CHROMOTHRIPSIS, AND RECURRENTLY ASSOCIATED ALTERATIONS IN A CONSECUTIVE COHORT OF 303 ADULT PATIENTS WITH NON M3 ACUTE MYELOID LEUKEMIA

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Introduction: Chromothripsis is a single catastrophic event in a full

chromosome or in a region, where the DNA pulverization is followed by activation of the DNA repair, resulting in a high number of breakpoints and copy number changes. These abnormalities could help define prognosis and therapeutic strategies in hematological diseases. The aim of the study is to establish the clinical impact of chromothripsis and discover new alterations associated with the chromosomal instability in Acute Myeloid Leukemia (AML).

Methods: Bone marrow specimens were collected at diagnosis in a cohort of 303 consecutive adult patients (pts) with non M3 AML (de novo and secondary). Chromosomal alterations were analyzed with classical cytogenetics and SNP Arrays (6.0 and Cytoscan HD, Affymetrix). The pts were screened for FLT3, NPM1 and TP53 mutational status, according to clinical routine, and survival data were collected. SNP Array data were analyzed by Nexus Copy Number™(BioDiscovery), the survival analysis was performed with Kaplan-Meier method using Mantel-Cox test. The frequence of alterations was compared with Fisher Exact Test.

Results: Twenty-three out of 303 pts (7.6%) showed chromothripsis events involving different chromosomes, with a random distribution pattern. The pts harboring chromothripsis presented a median age of 67.5 years, complex karyotype and exclusively high risk features according to ELN definition. Seventeen out of 23 pts with chromothripsis harbored a TP53 mutation, 2/23 had a single-copy loss of TP53, 2/23 were wild type (2/23 patients were not tested). TP53 mutation showed a significantly higher prevalence in patients with chromothripsis ($p<0.001$). Harboring chromothripsis defined a group of pts with poor prognosis compared with pts without ($p<0.001$), with a median survival of 2.9 months and 19.1 months respectively. Remarkably, chromothripsis defined the group with the worst prognosis even in the subset of patients with high risk features ($p<0.001$), with a median survival of 2.9 months and 17.3 months respectively (Figure 1). By comparing pts with and without chromothripsis, we identified significant Copy Number Alterations in the group of pts with the catastrophic event: RAD50, MARCH3, PRDM6, SSBP2, CDC23, HDAC3, CHD1, TBCA, LMNB1, and JMY were preferentially deleted ($p<0.001$); ZDPM2, RUNX2, RUNX1T1, FLT3, ERG, TTC3, and GPC6 were preferentially amplified ($p<0.001$); while the 5q loss was the most altered chromosomic region. The main pathways involved telomere and chromatin alteration ($p<0.001$).

Conclusion: Chromothripsis is a rare event in adult AML, although independently define a group of patients with poor prognosis. The strong association with TP53 alterations candidates TP53 deletion or mutation as the main permissive factor for chromotripsis event, preventing apoptosis in response of catastrophic chromosomal alteration.

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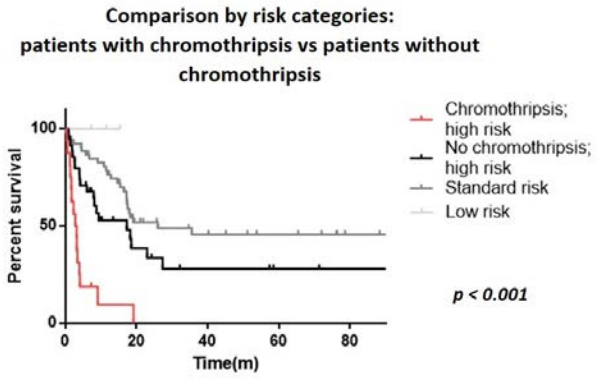

Figure 1.

P011

DELETERIOUS MUTATIONS IN UTX AS NEW MOLECULAR MARKER IN MYELOID MALIGNANCIES

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Introduction: Over the past decade, genomic studies have identified a number of novel and recurrent somatic mutations (DNMT3A, TET2, IDH1/2, ASXL1, EZH2, UTX) that affect epigenetic patterning in patients with myeloid malignancies, including myeloproliferative neoplasms, myelodysplastic syndrome and acute myeloid leukemia. Many of these mutations occur in genes with established roles in the regulation and maintenance of DNA methylation and/or chromatin modifications in hematopoietic stem/progenitor cells. The UTX gene (also known as KDM6A) encodes for a lysine demethylase specific for H3K27. The aim of this study was to evaluate frequencies of UTX somatic mutations and their correlation with the clinic-hematological characteristics in AML and in other myeloid malignancies patients.

Methods: We analyzed a total of 118 out of 850 (reached at the Institute Hematology "L. e A. Seràgnoli" from 2011 to 2016) AML and other myeloid malignancies patients at diagnosis: 91 AML/sAML, 15 MPN, 3 CML, 7 MDS and 2 CMML by TruSight Myeloid Sequencing Panel (Illumina). 25 patients (21,2%) had a normal karyotype, 25 (21,2%) presented one or two alterations, 26 (22%) had a complex karyotype, while for 42 patients (35,6%) no information about the karyotype was available. Sanger sequencing was applied in order to validate the candidate variants.

Results: The results were filtered by a coverage of minimum depth of 500 and allele frequency >5%. Variants already classified as SNP, silent and noncoding were removed. We identified 76 UTX variants (27 deleterious) in 22 out 118 (18.6%) patients with a mean of 3 alterations per patient (median 2 and range 1-8). UTX mutations were present at the C-terminus and the N-terminus of the UTX protein but mostly in the region adjacent to the JmjC domain required for UTX activity. Biological feature and clinical outcome will be presented. We analyzed the association between UTX and deleterious genes mutations in 16 patients. We identified a mean of 12,5 alterations per patients (median 13 and range 1-25), mainly TET2 (8 patients), DNMT3A (8 patients). In particular in 5 patients were present mutations EZH2 without mutually exclusive as reported. Only in 1 AML patients UTX mutations were present as sole event p. A340P (VF>5%). In other sAML patient we identified two non-sense mutations located before JmjC domain p.Q506X (VF>19%) and p. Q835X (VF>5%). UTX mutations with allele frequency >20% were selected to be validated by Sanger sequencing and we obtained 100% of concordance.

Conclusions: In conclusion, we observed that 1/3 of our AML carry a deleterious mutation in UTX. 1 patient have deleterious alterations only in UTX. Because of these observations we propose UTX as a potential marker in myeloid malignancies; in particular it might be interest to potential epigenetic treatment approaches.

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P012

CLINICAL OUTCOME AND EFFECT OF ALLO-SCT IN ADULT PATIENTS WITH DE NOVO OR AML-RELATED MYELOID SARCOMA. RESULTS FROM AN ITALIAN MULTICENTER SURVEY

D. Lazzarotto [1], A. Candoni [1], C. Filì[1], F. Forghieri [2], L. Pagano [3], A. Busca [4], G. Spinosa [5], E. Borlenghi [6], M. E. Zannier [1], E. Simeone [1], L. Melillo [7], F. Mosna 8, F. Lessi[9], R. Fanin[1]

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Introduction: Myeloid Sarcoma (MS) is a rare hematologic myeloid neoplasm that can involve any site of the body. It can occur as de novo extramedullary form or it can be associated with an AML, a MPN or a MDS at onset or at relapse (secondary MS). The rarity of MS does not enable prospective clinical trials and therefore a specific multicenter register can be very useful for the clinical and biological studies of this rare disease.

Patients and Results: We report the clinical characteristics and outcome of 53 histologically confirmed MS, diagnosed and treated in 9 Italian Hematological Centers in the last 10 years (2005-2015). The median age of these patients (pts) was 47 years (range 15-82) and 30 (56.6%) were male. There were 9/53 de novo extramedullary MS, 24/53 primary AML-related MS and 20/53 were secondary MS (median time of MS onset from the previous disease was 34.5 months, range 4–94). The most common extramedullary anatomic sites of disease were skin, lymph nodes, soft tissues, bone and testis. Treatment: 46/53 pts (86.8%) underwent a program of intensive chemotherapy (combined with radiotherapy in 16/46 cases) including FLAI, HDAC-IDA, HyperCVAD and MEC schemes, with a CR Rate of 43.5% (20/46). Twenty-four (52.2%) pts underwent Allo-SCT, 9 from an HLA-identical sibling donor, 2 from an haploidentical donor and 13 from a MUD. The median OS of the whole population was 16.7 months with no differences between de novo extramedullary MS and AML-related MS (p=0.71). OS probability at 5 years was 33,8%. Survival was significantly better in the pts that underwent intensive chemotherapy (median OS: 18.3 months vs 5.4 months, P=0,006). Furthermore, among the intensively treated pts, the survival was better in those pts that underwent Allo-SCT (median OS not reached vs 10.6 months, P=0,001), in pts with de novo or primary MS (median OS 20.4 mths vs 10.6 months of the secondary MS, P=0,012) and in the pts that achieved a CR after induction chemotherapy (median OS not reached vs 14.6 months, P=0,07) without differences between de novo extramedullary MS and AML-related MS (p=0.76). In multivariable analysis, Allo-SCT and Response to Induction therapy, were the only significant variables in predicting survival (P=0,002 and P=0,037, respectively). The median post-transplant OS of the Allo-SCT recipients was not reached after a median follow-up of 17.5 months and we observe a survival advantage in the patients who achieved a pre-transplant CR (P=0,042) and in the patients who developed a chronic GvHD after Allo-SCT (P=0,065).

Conclusions: The pts with de novo extramedullary MS result to have a similar unfavorable outcome than the patients with AML-related MS. These data outline the need of underwent an intensive therapeutic program that includes Allo-SCT, whenever possible, both in de novo extramedullary MS and in AML-related MS. The outcome after Allo-SCT is positively influenced by the development of chronic GvHD suggesting a Graft versus MS effect.

Myeloproliferative Disorders and Chronic Myeloid Leukemia 1

P013

ROLE OF HLA-G GENE POLYMORPHISM IN CHRONIC MYELOID LEUKEMIA

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Human Leukocyte Antigen-G (HLA-G) is a gene encoding for a tolerogenic protein expressed both in healthy tissues and solid tumors, promoting tumor immune-escape mechanisms and unfavourable outcomes. About 50 alleles encode for 17 full-length proteins, determining low or high secretion of soluble (s) HLA-G proteins which may thus inhibit the activity of cytotoxic and helper T lymphocytes, NK cells, neutrophils and antigen presenting cells. No studies have investigated its role in onset of chronic myeloid leukemia (CML), response to therapy with tyrosine kinase inhibitors (TKIs) and treatment-free remission (TFR) after TKI discontinuation. We investigated HLA-G polymorphism in 68 chronic-phase CML patients (mean age 63 years, range 27-88). The entire HLA-G gene was amplified by Long-Range PCR and sequenced using Next-Generation Sequencing (NGS) Nextera-Illumina technology with a 300bp paired reads protocol. The following alleles had a frequency higher than 1%: G*01:01 (G*01:01:01, G*01:01:02, G*01:01:03), G*01:03, G*01:04, G*01:05N, G*01:06. In comparison to HLA-G frequencies obtained from 1076 healthy subjects from the 1000Genome project, the frequency of the G*01:03 allele was significantly associated to CML (10,29% vs 4,46;p=0.001). Fifty-five patients (80.9%) were treated upfront with imatinib. Ten patients received second-line treatment with nilotinib (14.7%) and 3 patients with dasatinib (4.4%). Twenty-four of the 68 CML patients were enrolled in a TKI discontinuation study protocol. Among the remaining 44 patients, 8-year event free survival (EFS) and overall survival (OS) was 69.4% and 79.1%, respectively. Patients homozygous for G*01:01:01 or G*01:01:02 reported significantly lower EFS compared to patients with other allelic combinations (64.8% vs 82.2%; p=0.05). The 10-year cumulative incidence of deep molecular response (MR4) was 69.5%. Patients carrying the G*01:01:03 allele had significantly higher rates of MR4 (100% vs 65%), with earlier achievement of deep molecular response (median of 8 vs 58 months, p=0.001) (Figure). TKIs were discontinued in 24 patients after 2 years of confirmed MR4 at least. Overall, TFR was 57.7%. All patients with the G*01:01:03 allele remained in TFR (100% vs 49.5%, p=0.08). None of the patients homozygous for the G*01:01:01 or G*01:01:02 allele remained in TFR (0% vs 68.4%, p=0.023). These data suggest that HLA-G polymorphism may have a role in susceptibility to CML and be capable of predicting deep molecular response. In particular, HLA-G alleles associated to higher secretion of sHLA-G would seem to be associated with CML onset (G*01:03), and lower EFS and TFR (homozygosity for G*01:01:01 or G*01:01:02), possibly through a stronger inhibitory effect on the immune system in favor of tumor escape mechanisms. Conversely, the G*01:01:03 allele associated to lower levels of sHLA-G promotes achievement of MR4 and

TFR, suggesting increased cooperation of the immune system in CML cell clearance.

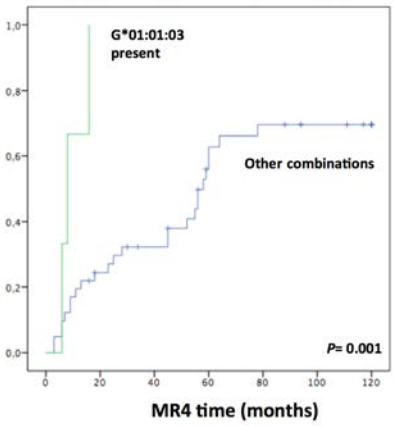

Figure 1.

P014

DEREGULATED EXPRESSION OF MIR-29A-3P AND MIR-494-3P AFFECTS TKI-SENSITIVITY OF CML LEUKEMIC STEM CELLS

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Introduction: Chronic myeloid leukemia (CML) is a clonal disorder that results from the malignant transformation of a very primitive hematopoietic stem cell (HSC). CML is characterized by a reciprocal t(9;22)(q34;q11) translocation, from which the BCR-ABL fusion oncogene arises. Currently, the recommended therapy for CML patients is the BCR-ABL tyrosine kinase inhibitor (TKI) imatinib mesylate. However, exposure to IM may not completely eliminate leukemic progenitors, in fact BCR-ABL-positive progenitors can be detected in patients in complete cytogenetic response (CCR). These cells are intrinsically resistant to TKI, therefore they represent the most likely candidate responsible for disease relapse. In the last few years, many studies have linked microRNAs (miRNAs) to leukemia pathogenesis. In order to understand whether miRNAs could play a role in the intrinsic resistance of CML LSCs to TKI, we perfomed miRNA expression profile (miEP) in CML LSCs.

Methods: miEP was performed using the Exiqon Human miRNome Panel. Deregulated miRNAs in CML were identified by comparing CML LSCs to normsl HSCs. In order to identfy miRNAs that are deregulated through a BCR-ABL-independent mechanism, K562 cells were treated with IM and miRNAs whose expression was not modulated were selected. To assess whether these miRNAs could affect IM sensitivity of CML cells, miRNA mimics were transfected in K562 cells and sensitivity to IM was measured by PI/AnnexinV staining.

Results: miEP analysis identified 32 deregulated miRNAs in CML LSCs. Among those, 5 miRNAs (miR-29a-3p, miR-365a-3p, miR-660-5p, miR-19b-2-5p, miR-204-5p and miR-494-3p) are deregulated in CML with a BCR-ABL-independent mechanism. Overexpression experiments showed that miR-29a-3p is able to confer IM resistance to K562 cells. By 3'UTR luciferase reporter assay, we identified TET2 as miR-29a-3p target. TET2 silencing was able to mimic the effect of miR-29a-3p overexpression, thus making K562 cells resistant to IM-induced apoptosis. On the other hand, between down-regulated miRNAs in CML LSCs, we found miR494-3p. Overexpression experiments showed that miR-494-3p is able to increase IM sensitivity in K562 cells. This effect is exerted through direct targeting of MYC, as demonstrated by 3'UTR luciferase reporter assay. Finally, MYC silencing in K562 cells induced a strong increase in IM-induced apoptosis, thus recapitulating the effects mediated by miR-494-3p overexpression.

Conclusions: We identified several miRNAs that are deregulated in CML LSCs through a BCR-ABL-independent mechanism. Our data show that the expression of such miRNAs could affect IM sensitivity of BCR-ABL-positive cells. In particular, the overexpression of miR-29a-3p confers IM resistance, whereas the overexpression of miR-494-3p increases IM sensitivity in K562 cells. Therefore CML LSCs might be able to escape IM treatment *in vivo* through the up-regulation of miR-29a-3p and the down-regulation of miR-494-3p.

P015

HIGH BCR-ABL/GUSIS LEVELS AT DIAGNOSIS ARE ASSOCIATED WITH UNFAVORABLE RESPONSES TO STANDARD DOSE IMATINIB

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Background: The approval of second-generation tyrosine kinase inhibitors (TKIs) for the first line treatment of Chronic Myeloid Leukemia (CML) has generated a need for early molecular parameters associated with inadequate responses to Imatinib Mesylate (IM).

Objective: We correlated quantitative determination of BCR-ABL transcripts at diagnosis with the outcome (defined according to the 2013 European Leukemia Net recommendations) of 272 newly diagnosed CML patients receiving IM 400 mg/die.

Methods; BCR-ABL transcripts were measured from peripheral blood samples drawn at diagnosis before patients received any pharmacological treatment using Real-Time Quantitative PCR (RQ-PCR). All molecular determinations were performed twice (in triplicates) on the same sample using either ABL or glucuronidase-beta (GUS) as reference genes. BCR-ABL values were then reported on the international scale (IS).

Results: With a median follow-up of 60 months, 65.4% of patients achieved an optimal response, 5.6% presented a response currently defined as "warning,"22.4% failed IM treatment and 6.6% switched to a different tyrosine kinase inhibitor because of intolerance to the drug. We recorded 19 deaths (6.9%), 7 (2.5%) attributable to

disease progression. We applied Receiver Operating Characteristic (ROC) curves to define BCR-ABL/GUSIS expression levels that would separate patients likely (i.e. below the threshold) or unlikely (i.e. above the threshold) to achieve multiple endpoints, namely: optimal response (OR), failure-free survival (FFS), event-free survival (EFS), transformation-free survival (TFS) and overall survival (OS). Employing the specific threshold calculated for each endpoint we found that high BCR-ABL/GUSIS levels at diagnosis were associated with inferior probabilities of OR ($p<0.001$), FFS ($p<0.001$) and EFS ($p<0.001$). Elevated BCR-ABL/GUSIS levels were also associated with higher rates of disease transformation to the accelerated phase or blast crisis ($p=0.029$) but not with OS ($p=0.132$).

Conclusions; High BCR-ABL transcripts at diagnosis measured by RQ-PCR employing GUS as a reference gene allow the identification of CML patients unlikely to benefit from standard dose IM that should be considered for alternative forms of treatment.

P016

ASSESSMENT OF BCR-ABL1 TRANSCRIPT LEVELS BY DIGITAL PCR (DPCR) IN CML PATIENTS WHO ACHIEVED A DMR (MR4.0, MR4.5 AND MR5.0) WITH TKIS (IMATINIB OR NILOTINIB) AND MAINTAINED THEIR DMR AFTER THE DISCONTINUATION OF THE TREATMENT

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The monitoring of BCR-ABL1 levels by quantitative PCR (qPCR) is essential for the management of CML patients treated with TKIs. Because of intrinsic limits, qPCR does not appear to be an optimal assay to select the best candidates to TKIs discontinuation. Indeed, up to 40% of CML patients treated with TKIs can achieve a Deep Molecular Response (DMR), but only 50% maintain a stable Treatment Free Remission (TFR) after therapy discontinuation. Digital PCR (dPCR), giving an absolute quantification of BCR-ABL1, is expected to be more sensitive and accurate than qPCR in the assessment of molecular Minimal Residual Disease (MRD). In a previous study presented at the 57th Annual Meeting of the American Society of Hematology, we quantified BCR-ABL1 transcript levels by dPCR in 116 CML patients treated with TKIs (imatinib, nilotinib or dasatinib) who achieved a major molecular response (MR3.0) or DMR (MR4.0, MR4.5 and MR5.0). We found that: a) BCR-ABL1 transcript levels were detectable by dPCR also in cases resulted undetectable by qPCR; b) 84% of deep responders (MR4.0, MR4.5 and MR5.0) fell under the value of 0.468 BCR-ABL1 copies/ul indicated by the ROC analysis as the value below which the patients with lower levels of MRD might be dissected (specificity=63.6%, sensitivity=84.3%; AUC=0,84). In this study, we aim at analyzing BCR-ABL1 transcript levels by dPCR in CML patients who achieved and maintained a stable DMR after TKIs discontinuation in order to evaluate if a more accurate quantification of BCR-ABL1 transcript levels may be more suitable to select patients for therapy discontinuation. dPCR was performed on a QuantStudio 3D Digital PCR System (Life Technologies) by using a TaqMan-MGB probes targeting the BCR-ABL1 transcript. Samples were obtained from CML patients treated with TKIs who maintained stable MR on time-checks planned for MRD monitoring by standard qPCR performed according to the last International Guidelines. The analysis by dPCR and qPCR were performed on 83 cases with stable DMR (MR4.0= 37 cases; MR4.5= 34 cases; MR5.0 = 12 cases) after giving informed consent. Twelve out of 83 DMR patients (15%) discontinued the therapy. Among them, 3 patients (25%) lost DMR and showed dPCR values > 0.468 BCR-ABL1 copies/ul (previously described as cut-off for a deep MRD), while 9 (75%) maintained a stable DMR and all of them fell under the value of 0.468 BCR-ABL1 copies/ul. These latter patients were stratified in different DMR classes by qPCR and all of them had undetectable level of BCR-ABL1 detected by qPCR. Thanks to its sensitivity, dPCR is able to give a more accurate quantification of BCR-ABL1 transcript levels, particularly in those patients with undetectable levels of BCR-ABL1 transcript as assessed by qPCR. Our preliminary results suggest that TFR can be correlated to the maintenance of dPCR values < 0.468 BCR-ABL1 copies/ul. [Acknowledgments: EuropeanLeukemiaNet, EUTOS, BCC "Pompiano e Franciacorta" and Cofin 2009].

P017

HDAC4 SILENCING SENSITIZES RESISTANT CML CELLS TO IMATINIB MESYLATE TREATMENT

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Introduction: Additional genetic and epigenetic changes in BCR-ABL-positive cells are often associated with inferior responses to Imatinib Mesylate (IM). Histone DeACetylases (HDACs) are a group of enzymes that modulate the acetylation of both histone and non-histone proteins involved in the regulation of gene expression, cell proliferation and cell death. Several studies have indicated that the combination of IM with HDAC inhibitors sensitizes both CML cell lines and CD34+ progenitors of IM-resistant patients to apoptosis.

Aim of this study was to determine if inactivation of the BCR-ABL kinase affects the expression and the catalytic activity of multiple HDACs in CML cells sensitive or resistant to IM in order to evaluate a possible therapeutic role for HDAC inhibitors in IM-resistant patients.

Methods: HDAC activity was evaluated employing a fluorescent assay kit. HDAC4 silencing was achieved by lentiviral infection of CML cells with specifically designed shRNA pools. The effects of IM and Apicidin were analysed by proliferation (MTS assay) and apoptosis assays (Annexin V/PI staining, caspase 3 activity).

Results: IM treatment of multiple CML cell lines increased total HDAC activity as compared to untreated cells. The same results were also observed when HL-60 cells were transduced with BCR-ABL while no difference in HDAC activity was detected in the empty vector transduced control. An immunoblot analysis revealed that variations in HDAC activity after pharmacological inhibition of BCR-ABL were associated with an increase in HDAC4 expression. Interestingly, HDAC4 silencing reduced HDAC activity after exposure of CML cells to IM. When we repeated these experiments in K562-R and LAMA84-R resistant to IM, we found that these cells displayed higher HDAC4 expression as compared to their IM-sensitive counterpart. Indeed, HDAC4 silencing restored IM response and determined an increase of their apoptotic rate. To

address the possible clinical implications of our results, we employed the HDAC4 inhibitor Apicidin. We found that the combination of IM and Apicidin enhanced the apoptotic rate of both IM-sensitive and IM-resistant CML cells. To further verify the efficacy of the combination of IM and Apicidin, we performed additional experiments employing CD34+ cells isolated from both IM-responsive and IM-resistant patients. We observed that the two-drug association determined a significant increase in cell death in IM-resistant but not in IM-sensitive CD34+ progenitors cells.

Conclusions: Our results suggest that BCR-ABL tyrosine kinase negatively modulates HDAC enzymatic activity mostly by reducing HDAC4 expression. Furthermore, strategies aimed at reducing HDAC4 levels in IM-resistant cells may represent a potential therapeutic option for CML patients unresponsive to IM treatment.

P018

ROLE OF AURORA KINASE A (AURKA) IN IMATINIB RESISTANCE

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Introduction: AURKA has a CENTRAL role in CML genomic instability. Its constitutive activation associated with the BCR-ABL1 TK activity promotes the progression of mitosis irrespective of the integrity of replicated DNA. Published studies have proven the therapeutic advantage of AURK inhibitors in CML patients either responsive or resistant to imatinib (IM). Such AURK inhibitors potential has been ascribed to their inhibitory activity on p210 TK activity. In this study, we have focused on the specificity and mechanisms of action of AURK inhibitors in CD34+ hematopoietic cell fraction.

Methods: We investigated the events leading to AURKA deregulation in the presence of BCR-ABL1. To this purpose, the CD34+ hematopoietic cell fractions were investigated for the phosphorylation levels of AURKA and other pro-survival components of its signalling pathways:

• FoxM1, a proliferation-associated transcription factor implicated in the advantage of clonal hematopoiesis over the normal counterpart, particularly in the leukemic stem cell (LSC) compartment;

• Plk1, a ser-thr kinase involved in M/G1 progression;

CD34+ cells were isolated from bone marrow samples of 10 CML patients at clinical diagnosis. CD34+ cells from peripheral blood of healthy donors were used as normal controls. RT-PCR, Western Blotting and Immunoprecipitation were performed to investigate gene expression and protein interactions.

Results: Our results proved a FOXM1 increment associated with IM resistance. An IM-resistant K562 cell line generated in our lab exhibited FoxM1 over-expression and hyper-phosphorylation contingent upon the upstream activation of AURKA and Plk1. In fact, in IM-resistant K562 cells, both Plk1 inhibition by volasertib (1∓M) and AURKs inhibition by danusertib (1∓M), activated a significant increment of apoptotic cell death compared to parental cell line. AURKA, FoxM1 and Plk1 involvement in IM resistance was confirmed in mononuclear cell fraction from bone marrow samples of 3 CML patients who developed IM resistance independent from BCR-ABL1 point mutations. Interestingly, the putative BCR-ABL1+/CD34+ compartment, which is neither dependent on BCR-ABL1 TK for proliferation and survival nor killed by IM and second generation inhibitors, showed a hyper-phosphorylation of AURKA and a consequent overexpression and hyper-activation of FoxM1 and Plk1. Moreover, clonogenic assays performed by using CD34+ progenitors from 5 CML patients at diagnosis showed that volasertib and danusertib are able to reduce the clonogenic potential of the CD34+ compartment to a much greater extent compared to 1st and 2nd generation TKIs.

Conclusions: The BCR-ABL1+ compartment provides a sanctuary for disease relapse upon drug withdrawal as well as a putative source of drug-resistance. We have identified a new signaling pathway involved both in drug resistance and in CD34+ cell survival. Our data open the route to novel therapeutic approaches worth exploring in order to overcome drug resistance.

P019

BCR-ABL DOUBLING-TIME AND HALVING-TIME MAY PREDICT CML RESPONSE TO TYROSINE KINASE INHIBITORS

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Introduction: The discovery of BCR-ABL tyrosine kinase inhibitors (TKIs) has significantly improved the natural history of Chronic Myeloid Leukemia (CML). TKI therapy requires molecular monitoring by RQ-PCR in order to evaluate treatment response and provide timely interventions for patients (pts) failing to achieve the desired outcomes. Aims of the study were: 1. to determine whether BCR-ABL doubling-times (DTs) could distinguish inconsequential rises in BCR-ABL transcripts from resistance to the assigned treatment; 2. to investigate if BCR-ABL halving times (HTs) could identify pts likely to obtain deep molecular responses.

Methods: We conducted a retrospective study examining a total of 377 chronic phase CML. 315 received Imatinib Mesylate (IM) as first line treatment while the remaining 62 received second-generation (2G) TKIs (17 Dasatinib and 45 Nilotinib). For this analysis we considered only patients with BCR-ABLIS levels <10% or >0.0000 in two consecutive analyses with no less than 10.000 ABL copies. The formulas employed to calculate DTs and HTs were as previously reported by Branford et al. (Blood 2012, 119: 4264-4271 and Blood 2014, 124: 511-518).

Results: We classified 315 IM-treated patients in three groups:

subjects that failed IM (n=34); subjects that lost a previously achieved MR3 but maintained a complete cytogenetic response (CCyR) (n=77); subjects with a confirmed rise in their BCR-ABL transcripts without MR3 loss (n=204). Short DTs were observed in pts failing IM (median 34.85 days). On the contrary, long DTs were found in subjects loosing a MR3 while maintaining a CCyR (median 52.0 days), and in individuals with a rise in BCR-ABL transcripts without MR3 loss (median 60.5 days). Moreover, we found that DTs registered in individuals failing 2G TKIs (n=14) as second line treatment, were shorter than those observed for IM-resistant pts (n=34) (28.5 versus 34.8 days; p=0.0284). BCR-ABL HTs were lower in 39 subjects with MR3 after 6 months of IM as compared to 48 individuals without MR3 (21.7 vs 42.7; p=0.0017). Furthermore, after 12 months of IM, short halving times occurred in 89 pts with MR3 as compared to 18 individuals without MR3 (30.6 vs 97.3 days; p<0.001). Similar results were found in pts receiving 2G TKIs as first line therapy. After 6 months of therapy, the 25 subjects with MR3 displayed a shorter HT than the 11 individuals without MR3 (22.5 vs 43.5 days; p=0.003). Likewise, pts in MR4 after 12 months presented a shorter HT as compared to individuals without MR4 (42.8 vs 91.6 days; p=0.019).

Conclusions: The length of BCR-ABL transcript DTs is a reliable tool that records major differences in CML kinetics according to the clinical context and accurately distinguishes pts failing TKIs, from those with inconsequential BCR-ABL increases. Likewise,

BCR-ABL HTs may identify patients likely to achieve deep molecular responses that may be considered for treatment discontinuation.

P020

PREDICTORS OF STABLE DEEP MOLECULAR RESPONSE IN CHRONIC PHASE CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH DASATINIB OR NILOTINIB AFTER IMATINIB FAILURE

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Background. Front-line treatment of newly diagnosed Chronic Myeloid Leukemia (CML) patients with 2nd generation tyrosine kinase inhibitors (2G-TKI) dasatinib (DAS) or nilotinib (NIL) resulted in higher rates of deep molecular response (MR) as compared to imatinib. Very little is known about rates and stability of deep MR when 2G-TKI are used after imatinib failure.

Methods. We analyzed 127 consecutive chronic phase CML patients treated with imatinib 400 mg daily as first-line therapy and then switched to 2G-TKI for resistance or intolerance. Patients progressing to advanced phases before switch were excluded. Deep molecular response (MR4) was defined as BCR-ABL ratio ≤0.01% or undetectable disease with ≥10,000 ABL copies. Patients with MR4 lasting ≥ 2 years, ongoing at the last contact, and with at least a Q-PCR test every 6 months were defined as stable MR4. Patients with any sample >0.01% BCR-ABL after the achievement of MR4 were defined as unstable MR4. Age, sex, Sokal and EUTOS risk score, type of BCR-ABL transcript, duration of imatinib, reason for switch to 2G-TKIs and early molecular response to 2G-TKI have been examined for the association with stable MR4. Frequencies were compared by Fisher's exact test. Univariate and multivariate regression analysis were performed using the competing risk model of Fine and Gray.

Results. Median age at diagnosis was 55 years (range 20-88) and median duration of imatinib treatment was 19 months (range 1-115). Patients resistant (n=89; 70%) or intolerant (n=38; 30%) to imatinib were switched to DAS (n=82; 64.5%) or NIL (n=45; 35.5%). Thirty-six patients were resistant or intolerant to their first 2G-TKI and were switched to 3rd-line NIL (n=20), DAS (n=12), bosutinib (n=2), or ponatinib (n=2). At a median follow-up of 52 months after switch to 2G-TKI (range 6-126), best deep MR to 2G-TKI was: no MR4 in 57 patients (45%), unstable MR4 in 28 patients (22%; 24 with 2nd line and 4 with 3rd line treatment), and stable MR4 in 42 patients (33%; 37 with 2nd line and 5 with 3rd line treatment). Five-year cumulative incidence of stable MR4 was 28.7% (95%CI: 18.9-37.3%). Age, sex, risk scores at diagnosis, type of BCR-ABL transcript, duration of imatinib and type of 2G-TKI were similar between patients with or without stable MR4 (table). Predictors of stable MR4 were reason for switch to 2G-TKI (intolerance vs resistance HR 0.41, 95%CI: 0.22-0.79; p=0.007) and 3-month BCR-ABL level after 2G-TKI start (≤10%IS vs >10%IS HR 0.08, 95%CI: 0.01-0.59; p=0.01). Three stable MR4 patients attempted discontinuation and are presently in treatment-free remission phase at 4, 24 and 29 months after 2G-TKI stop, respectively.

Conclusions. In this real-life experience, long-term use of 2G-TKI in ≥2nd line of treatment after imatinib failure resulted in more than half of patients achieving the "safe haven" of deep MR, with around 60% of them in stable MR4, a prerequisite for discontinuing treatment.

Table I. Characteristics of patients and frequency of MR[4] (unstable or stable)

Characteristics	No MR[4] (n=57)	Unstable MR[4] (n=28)	Stable (≥2 yrs) MR[4] (n=42)	p
Sex (male / female)	34 / 23	19 / 9	22 / 20	0.43
Age, median (range)	59 (22-88)	55 (20-80)	55 (23-78)	0.77
Sokal risk				
- low	21 (37)	12 (44)	16 (38)	
- intermediate	22 (39)	14 (50)	18 (43)	0.56
- high	11 (19)	1 (3)	7 (17)	
- not evaluable	3 (5)	1 (3)	1 (2)	
EUTOS risk				
- low	50 (88)	22 (79)	39 (93)	0.33
- high	2 (3)	2 (7)	2 (5)	
- not evaluable	5 (9)	4 (14)	1 (2)	
BCR-ABL transcript				
- b2a2	26 (46)	15 (54)	19 (45)	
- b3a2	18 (31)	10 (36)	18 (43)	0.74
- b2a2/b3a2	7 (12)	1 (3)	3 (7)	
- other	6 (11)	2 (7)	2 (5)	
Duration of imatinib				
- <12 months	24 (42)	7 (25)	13 (31)	0.27
- ≥12 months	33 (58)	21 (75)	29 (69)	
Reason for switch				
- intolerance	10 (17)	11 (39)	17 (41)	**0.019**
- resistance	47 (83)	17 (61)	25 (59)	
Type of 2G-TKI				
- dasatinib	39 (68)	14 (50)	29 (69)	0.20
- nilotinib	18 (32)	14 (50)	13 (31)	

P021

MONOCYTIC MYELOID DERIVED SUPPRESSOR CELLS (M-MDSC) AS PROGNOSTIC FACTOR IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Background: Recently, we and another group demonstrated that Myeloid suppressor cells play an important role of immune escape in CML patients. Aims: Investigating the effect of the tyrosine kinase inhibitors (TKI) therapy on MDSC and possible correlation with clinical response.

Methods: CML patients at diagnosis (n=30) and during TKI treatment were enrolled in this study. Eighteen patients were treated with imatinib (IM), 13 with nilotinib (NIL) and 12 with dasatinib (DAS). MDSCs were also analyzed in 20 healthy donors (HD). Granulocytic MDSCs (G-MDSCs) were identified as CD11b+ CD33+CD14- HLADR- cells while monocytic MDSCs (M-MDSCs) as CD14+HLADR by cytofluorimetric analysis. Their immunosuppressive activity was tested through incubation with autologous CFSE+T cells. Exosomes were isolated from CML serum at diagnosis (n=5) by sequential ultracentrifugation.

Results: G-MDSCs and M-MDSCs percentages in CML patients were greater than HD (p<0.0001). Both isolated subpopulations showed expression of BCR/ABL and were able to inhibit T cells proliferation in comparison to positive control (p=0.005; p<0.0001). No suppressive effect was observed in co-cultures with G-MDSC and M-MDSC obtained from HD. In addition, M-MDSC percentage correlated with BCR/ABL transcript levels in patients at diagnosis (r=0.579, p=0.0004). Evaluating the effect of TKI therapy on MDSC levels, we found that both IM, NIL and DAS induced a significant reduction of G-MDSC percentage at 6 months and 12 months of treatment. The levels of M-MDSCs significantly decreased only after DAS therapy (from 33.6±19% to 6.8±12.6%

at 6 months, p=0.014 and 11.4±12.3% at 12 months, p=0.008). M-MDSC reduction was also present but did not reach statistical significance after IM treatment and after NIL. Moreover, we found that in DAS, but not in IM or NIL treated patients, a correlation between percentage of Major Molecular Response (MMR) and number of persistent M- MDSCs was found. A significant difference was calculated comparing M-MDSC levels in the MMR group (n=6) versus no MMR (n=6) at 12 months (p=0.008). To evaluate if leukemic cells are able to expand MDSC releasing soluble factors or exosomes, we incubated monocytes obtained from HD with sera or exosomes from CML patients at diagnosis or HD. M-MDSCs percentage significantly increased only in conditions with CML serum (29±13%; p=0.0006) or exosomes (8±2.8%; p=0.01). No effect was observed on G-MDSC percentage.

Conclusions: Therapy with TKI reduces the percentage of MDSCs and levels of the monocytic subset correlates with MMR value in patients treated with dasatinib, suggesting their importance in clinical investigation as prognostic factor. Moreover, our data suggest the possible development in CML patients of a circuit primed by tumor cells that, through the release of soluble factors and exosomes, are able to expand M-MDSCs, creating an immunotolerant environment that results in T cell anergy and facilitates tumor growth.

P022

DROPLET DIGITAL PCR ASSAY FOR QUANTIFYING OF CALR MUTANT ALLELIC BURDEN IN MYELOPROLIFERATIVE NEOPLASMS

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Introduction. Calreticulin (CALR) gene mutations (CALRmut) have recently been discovered in about 20-35% of patients affected by essential thrombocytemia (ET) and primary myelofibrosis (PMF), increasing the proportion of patients with myeloproliferative neoplasms (MPNs) characterized by a specific molecular marker. Moreover, the identification of CALRmut has relevant prognostic and clinical implications, as both ET and PMF patients bearing CALRmut have higher platelet counts, lower hemoglobin and better overall survival than JAK2 mutated patients. Several molecular assays and targeted next generation sequencing have been developed to detect the most frequent CALRmut (type 1 consisting of a 52-bp deletion, and type 2, of a 5-bp insertion). These techniques are useful for identifying CALRmut at the diagnosis of MPNs, but they are not suitable for minimal residual disease (MRD) monitoring, since the maximum sensitivity is about 1%. In this paper, we report a droplet digital PCR (ddPCR) assay developed for the absolute quantification of CALR type 1 and 2 mutations and analyze a cohort of 57 JAK2V617F negative MPNs patients.

Methods. ddPCR experiments were performed using the QX-200 instrument (Biorad). Specific primers and probes were designed for both type 1 and type 2 mutation. Each assay contains a single pair of primers and two competitive probes, one specific for the wild-type allele, and one specific for the mutant allele. The analysis of results was performed using QuantaSoft software version 1.7.4. CALRmut load in each sample was expressed as Fractional Abundance (FA: mutant allele/ mutant allele + wild type allele).

Results. The ddPCR analysis was successfully performed in all 57 MPNs cases bearing CALR type 1 or 2 mutations. The CALRmut allelic burden resulted heterogeneous ranging from a minimum of 13.8% to a maximum of 51% in ET and from a minimum of 34.5% to a maximum of 51.3 % in PMF patients. The median CALRmut allelic burden at diagnosis resulted significantly different between PMF and ET (47.9% vs 43.8%, p = 0.008). Sequential evaluations by ddPCR experiments were performed in a few patients to monitor the CALRmut load during treatment. CALRmut load at diagnosis was 15.8% and 48% in two ET patients. The former patient was treated with interferon-↦ ⌟IFN-↦) and after five years from diagnosis the FA was 7.7%. The latter was also treated with IFN-↦ and after two years from diagnosis the CALRmut load was 14.7%. Both patients had stable disease and a well controlled platelet count.

Conclusions. Although the importance of the CALR allelic burden determination has not yet been defined at the disease onset, the utility of and need for a sensitive method are unquestionable for the purposes of molecular disease monitoring. Our CALRmut ddPCR assay reaches a sensitivity of 0.01% and offers a precise molecular method to perform absolute quantification of CALR mutations both at diagnosis and during the follow-up of TE and PMF patients.

Chronic lymphocytic leukemia and Chronic Lymphoproliferative Disorders 1

P023

ASSOCIATION OF REDOX SIGNALING HYPERSENSITIVITY WITH FAVORABLE PROGNOSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Introduction. Chronic lymphocytic leukemia (CLL) patients exhibit a variable clinical course. The B-cell receptor (BCR) signaling is a key determinant of heterogeneous clinical behavior of CLL and is a target for therapeutic interventions. Endogenously produced H2O2 is thought to fine-tune the level of BCR signaling by reversibly inhibiting phosphatases. However, relatively little is known about how CLL cells sense and respond to such redox cues In this study, we compared BCR signaling responses to H_2O_2 in prognostic groups of CLL patients.

Methods. The phosphorylation levels of five proteins downstream of the BCR signaling, namely SYK, NF-κB p65, ERK, p38 and JNK, were analyzed at the single-cell level in 26 CLL cell samples using phospho-specific flow cytometry. Protein phosphorylation was measured in the basal condition and following stimulation with H_2O_2. B cells from healthy individuals were analyzed as controls. Time to first treatment (TTFT) was calculated from the date of diagnosis to the date of initial therapy. TTFT curves estimated using the Kaplan-Meier method for the respective groups of patients were compared using the log-rank test.

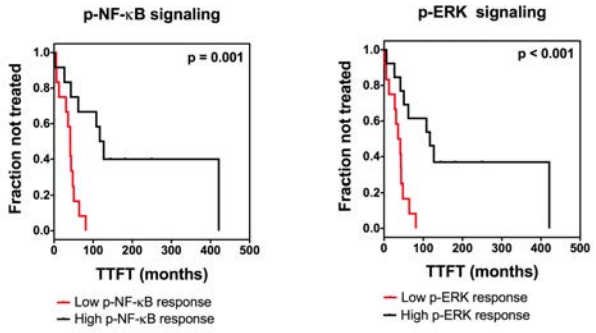

Figure 1. Kaplan-Meier curves of TTFT for subgroups of patients defined by response to H_2O_2.

Results. Stimulation with H_2O_2 induced a statistically significant increase in phosphorylation of all analyzed signaling proteins with the exception of SYK. Moreover, the extents of responses to H2O2 were significantly higher in CLL than normal B cells for all signaling proteins but SYK. Comparison of H_2O_2 signaling response in prognostic groups of patients defined by IGHV mutational status, CD38 or ZAP-70 expression, showed that median phosphorylation response of ERK to H_2O_2 was significantly higher in the patient subset with mutated IGHV (M-CLL) (P=0.031). Kaplan-Meier curves showed statistically significant slower progression (longer TTFT) in patients with higher p-ERK and p-NF-κB p65 responses to H2O2, indicating that lower responsiveness of these signaling proteins to H2O2 correlated with more rapid progression [median TTFT was 41.6 and 115 months for patients with lower and higher NF-ŒʃB p65 responsiveness to H_2O_2, respectively (log-rank test P=0.001); median TTFT was 38.7 and 117.0 months for patients with lower and higher ERK responsiveness to H_2O_2, respectively (log-rank test P=0.0008)] (Figure 1). Interestingly, the ability of H2O2 responsiveness signaling to define prognostic groups is comparable to that of IGHV mutational status (log-rank test P=0.0003).

Conclusions. This study reveals that a novel H_2O_2 signaling response distinguishes a prognostic group of CLL patients with favorable prognosis. Specifically, higher H_2O_2 responsiveness of ERK or NF-κB is predictive of longer TTFT, thus highlighting ERK and NF-κB as biologically and clinically relevant signaling nodes in CLL.

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P024

THE EFFECT OF IL-6 AND IL-15 IN LARGE GRANULAR LYMPHOCYTE LEUKEMIA DEPENDS ON STAT MUTATIONS AND LEUKEMIC CELLS IMMUNOPHENOTYPE

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Introduction: Large Granular Lymphocyte Leukemia (LGLL) is a disorder characterized by the chronic, clonal expansion of lymphocytes with cytotoxic activity. According to LGL immunophenotype two different types of disease have been defined: T-LGLL characterized by CD3+/CD8+ or by CD3+/CD4+ lymphocytes and Chronic Lymphoproliferative Disorder of Natural Killer cells (CLPD-NK) characterized by CD3-/CD16+ cells. The etiology of this disease is largely unknown. Several data indicate that LGL proliferation is maintained through the activation of different pro-survival signaling pathways (JAK/STAT, PI3K/AKT, MAPK/ERK), maintained in a pro-inflammatory milieu rich of IL-6 and IL-15, two key cytokines in LGLL pathogenesis. Recently, the presence of STAT mutations has been discovered in 30% of patients affected by LGLL. These mutations induce a constitutive activation of JAK/STAT pathway which contributes to LGL clone expansion. The aim of this study is to evaluate the role of IL-6 and IL-15 in the different variants of LGL leukemia, defined according to the LGLs immunophenotype and STAT mutations.

Methods: One hundred and six patients with T-LGLL and 21 with CLPD-NK were studied. Peripheral blood mononuclear cell (PBMC) immunophenotype was analyzed by flow cytometry. For molecular analysis, LGLs were purified by microbeads or FACSAria cell sorting. Mutational study was performed on DNA samples by Sanger sequencing and by amplification Refractory Mutations System. Expression analysis of IL-6 and IL-15 was obtained by Real Time PCR. By PBMC culture we evaluated the effect of IL-6 or IL-15 stimulation: apoptosis by Annexin V test and the activation of STAT3, STAT5b, AKT and ERK by western blotting analysis. Statistical analysis was performed by Wilcoxon-Mann-Whitney test and Kruscall-Wallis test.

Results: Considering LGL's immunophenotype (T-LGL CD4+, CD8+ or NK) and STAT mutations (STAT3 and STAT5b), we distinguished 6 classes of patients. Our results showed that IL-15 was highly expressed in the aggressive form of LGL leukemia, whereas IL-6 was equally expressed in all different classes of patients. Moreover, IL-15 had a greater effect on cell survival with respect to IL-6 for all patients' subsets. In particular, IL-15 activated different pro-survival pathways, including MAPK/ERK, PI3K/AKT and JAK/STAT whereas IL-6 was specific only for JAK/STAT signalling. We observed that IL-6 activity was more evident on STAT3 wild type patients, whereas the presence of STAT3 mutation confered more responsiveness to IL-15. On the contrary, considering STAT5b protein, we showed that this isoform responded only after IL-15 stimulus and it is insensitive to IL-6 effect both in wild-type and in presence of mutations.

Conclusions: By confirming the role of IL-6 and IL-15 in LGL leukemia, we provided evidence that these cytokines could represent a potential therapeutic target, according to the different role reported between patients' subsets.

P025

EXPRESSION OF FAK AND ITS INVOLVEMENT IN THE PROGRESSION OF B CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: B cell Chronic Lymphocytic Leukemia (CLL) is a disorder characterized by the accumulation of clonal CD19+CD5+ B lymphocytes, due to uncontrolled growth and resistance to apoptosis. Although the prognosis and clinical outcome has been improved by recent innovative therapies, CLL still remains an incurable disease. Since signaling events downstream the B Cell Receptor (BCR) engagement are important for the progression of B cells, BCR signaling has been investigated in CLL with the ultimate goal to design new agents to specifically treat this disease. We demonstrated that Lyn, one of the first kinases involved in BCR signaling pathway, is over-expressed, constitutively active and anomalously distributed in malignant B cells, as compared to normal B lymphocytes. The Focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase, is the primary enzyme involved in the engagement of integrins and assembly of Focal Adhesion. FAK is regulated primarily through tyrosine phosphorylation by Lyn after BCR engagement and was found to be over-expressed in many kinds of human cancers. However, a down-modulation of FAK expression and its association to poor prognosis have also been reported. The aim of this study was to investigate the role of FAK in CLL patients.

Methods: Blood samples were collected from 50 CLL patients and 5 healthy controls. Peripheral blood B cells were purified using the RosetteSep for human B cells isolation kit. The samples that were used had at least 95% of normal CD19+ or neoplastic CD19+CD5+ cells. Protein levels were evaluated by Western blotting (Wb) analyses.

Results: Wb analyses of FAK revealed no significant difference in protein expression between patients and controls (0.54±0.65 vs 0.85±0.32, p=ns). Considering the high standard deviation in CLL, we divided our patients into 2 groups: i) patients with levels of FAK comparable to the normal (n=22, FAK High: 0.95± 0.58) and related to positive prognostic markers ($p<0.05$); ii) patients with low expression (n=28, FAK Low: 0.28± 0.25) related to the negative prognostic markers ($p<0.05$). We found that patients (n=6) who had an indolent course and were responsive to the standard R-FC treatment, showed normal expression of this kinase already at diagnosis. By contrast, patients (n=6) with an aggressive disease, had a lower expression of FAK, that was further down-modulated during the disease progression. FAK and p53 are reciprocally regulated by a loop with a negative feedback; we assessed in 9 patients the presence of p53 in relation to FAK expression, finding that this mechanism was preserved only in 3 patients and neither mutations of TP53 gene nor recurrent genomic aberrations seemed to explain the loss of this regulation loop observed in some patients.

Conclusions: From these data we propose that FAK down-modulation could be considered as a new marker of poor prognosis and as a putative predictor for high-risk subgroups of CLL, even in early-stage disease.

P026

DOMINANT CYTOTOXIC NK CELL SUBSET WITHIN CLPD-NK PATIENTS RECOGNIZES A MORE AGGRESSIVE NK CELL PROLIFERATION

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Background: NK cells represent a class of innate lymphocyte with cytotoxic functions with CD3-/CD16+/CD56+ phenotype. According to CD16 and CD56 expression, CD56high/CD16dim/neg NK cells and CD56dim/CD16high NK cells subsets can be distinguished; moreover, CD57 and CD62L expression allow to identify CD56dim/CD16high/CD57+/CD62L- subtype of NK cells with memory properties. Chronic Lymphoproliferative Disorder of NK Cell (CLPD-NK) is characterized by persistent expansion of NK cells, with neutropenia representing the major feature of the disease. Only few information are available about the pathogenesis of CLPD-NK but a constitutive JAK-STAT pathway activation is hypothesized to be involved because of discovery of somatic STAT3 mutations in about 30% of patients. Using flow analysis, the aim of the present study was to identify different NK cell subsets of CLPD-NK patients with different phenotypic, clinical and molecular characteristics.

Methods: NK cells from 24 patients affected by CLPD-NK were analysed by flow for CD3, CD16, CD56, CD57 and CD62L expression. All patients showed KIR restriction, as a surrogate diagnostic criterion. Finally, STAT3 mutation analysis of exon 21 was performed with Sanger sequencing and ARMS PCR. Patients were also evaluated for clinical features and treatment requirement.

Results: According to CD16 and CD56 expression in our series of CLPD-NK patients, 13/24 (54%) were characterized by prevalent CD56neg/dim/CD16high NK population, 9/24 (38%) by CD56dim/CD16neg NK population and only 2 out of 16 (8%) by CD56high/CD16neg NK population. Interestingly, among CD56neg/dim/CD16high subgroup, patients with prevalence of CD56neg/dim/CD16high/CD57-/CD62L- cytotoxic NK cells (5/13, 38%) were characterized by symptomatic disease, according to the presence of severe neutropenia (5/5, 100%), and treatment need (3/5, 60%). On the other hand, the 8 patients (62%) characterized by expansions CD56neg/dim/CD16high/CD57+/CD62L- memory NK cells and the other subsets of patients, presented a more indolent behaviour of disease with no severe neutropenia and treatment need. Finally, STAT3 mutations analysis was performed and we found two (2/24, 8%) mutated patients (one by Sanger sequencing and one by ARMS PCR); all mutated patients were in CD56neg/dim/CD16high/CD57-/CD62L- cytotoxic NK cells subsets, while the other subgroups of patients resulted wild type.

Conclusions: Through NK cells subsets flow analysis, discrete subtypes of CLPD-NK can be identified. Independently from KIR expression, patients characterized by CD56neg/dim/CD16high/CD57-/CD62L- cytotoxic NK cells expansion represent a different phenotypic subgroup of patients characterized by more symptomatic disease and the presence of STAT3 mutation, suggesting a more aggressive proliferation of NK cells.

P027

INHIBITION OF USP7 INDUCES SELECTIVE CANCER CELL DEATH IN CHRONIC LYMPHOCYTIC LEUKEMIA THROUGH PTEN AND INDEPENDENTLY FROM P53 STATUS

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Introduction: Standard immune-chemotherapy allows to achieve 60-70% response rate in CLL patients, but complete remission remains uncommon. Unsatisfactory therapeutic options still exist for higher risk groups, and in particular those harboring TP53 mutations or deletion.

Methods: Primary CLL cells were enriched in CD19+ fraction using the Milteny anti-CD19 kit and were used to investigate USP7 levels. P5091 (USP7 inhibitor) efficacy was tested in CLL cell lines and patients. Proliferation and apoptosis were evaluated respectively with CTG technology and Annexin V-FITC/Propidium PE detection by flow cytometry. The specificity of the USP7 inhibitor was verified with a pool of 5 different siRNAs able to efficiently silence USP7.

Results: Our data show that the de-ubiquitinase USP7 is aberrantly expressed in CLL. In particular, USP7 is over- expressed in about 70% of CLL CD19+ lymphocytes, both at the mRNA and protein levels. We also analyzed USP7 expression levels in an expansion cohort of a publicly available CLL patients (n=217) and 12 normal samples, where USP7 was over-expressed in CLL when compared to normal samples (****p<0.0001). We proved that USP7 is regulated at post-transcriptional level by miR-338-3p and functionally activated by Casein Kinase 2 (CK2) through phosphorylation at serine 18 residue. USP7 inhibition by P5091 as well as by specific siRNA, induces cell growth arrest and apoptosis mediated by the restoration of the nuclear pool of PTEN. Notably in primary CLL cells, P5091 treatment strongly promoted apoptosis. Moreover, USP7 inhibitor treatment of primary CLL was associated with increases ubiquitination of endogenous PTEN and consequently PTEN relocalization into the nucleus. Strikingly, TP53 deleted CLL samples and cells lines were equally subject to P5091 apoptosis induction, confirming that the USP7 inhibitor acts in a p53 independent manner. These data showed potent activity of P5091 against primary CLL.

Conclusions: We demonstrate the efficacy of a small molecule inhibitor of USP7, P5091, *in vitro* in cell lines and *ex vivo* in primary CLL samples in a P53-independent manner. Our preclinical study therefore, supports the evaluation of USP7 inhibitor as a potential CLL therapy.

P028

THE DUAL ENDOTHELIN RECEPTOR ANTAGONIST MACITENTAN THWARTS SURVIVAL, HOMING AND ANGIOGENIC PROFILE OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Background. Endothelin 1 (ET1) is a peptide that mediates its action by activating two G-protein-coupled receptors, ETAR and ETBR. Chronic lymphocytic leukemia (CLL) cells secrete ET1 and express ETA and ETB receptors on the cellular surface. Macitentan, a double inhibitor of ETA and ETB receptors, induces direct apoptosis and overcomes resistance to chemotherapeutics in solid tumors. We aim at evaluating macitentan effects on CLL cells.

Methods. CD19+ CLL cells isolated from patients were incubated *in vitro* with macitentan 10 μM and then treated with 100nM ET1. In some instances, CLL cells were pre-incubated with BTK inhibitor ibrutinib 0.5 μM. We evaluated migration, adhesion to stromal and endothelial cells, B-cell receptor (BCR) signaling pathway and angiogenic profile of CLL cells. Moreover, the expression of ET1, ETAR and ETBR in CD49d+ CLL vs. CD49d- CLL as well as in CXCR4high CLL vs. CXCR4low CLL was measured in peripheral blood of CLL patients by flow cytometry. We also evaluated big ET1 level by ELISA in plasma samples from MBL, CLL at diagnosis and at first progression, in fludarabine resistant CLL and in CLL after ibrutinib treatment.

Results. ET1 acts as a chemotactic factor for CLL cells. The blockage of ET receptors impairs CLL migration to ET1. Moreover, macitentan cooperates with ibrutinib in disrupting CLL migration to chemokine gradients. Accordingly, we found that circulating CXCR4high CLL cells express higher levels of ET1 and ET1 receptors than CXCR4low fraction ($p<0.05$). ET1 signaling is involved in CLL adhesion, as inferred on the basis of the following observations: (i) macitentan leads to CLL detachment from stromal and endothelial cell supports; (ii) ET1 stimulation promotes the phosphorylation of FAK, GSK3β, and AKT; (iii) ET1 increases CD49d expression in CLL and (iv) higher levels of ET1 and ET receptors characterize CD49d+ CLL fraction in peripheral blood. We also found that BCR and CD40 activation leads to ET1 increase in CLL cells. Macitentan disrupts BCR signaling and survival, synergizing with ibrutinib effect. Moreover, ET1 stimulation promoters HIF1α accumulation in CLL cells, leading to an improvement of pro-angiogenic features of leukemic cells. In plasma samples, big ET1, the precursor of ET1, was detectable at variable levels ranging from 0.6 to 67 pg/mL. Mean big ET1 levels were equal to 2 pg/mL in MBL patients, 3.5 pg/mL in CLL at diagnosis, 7.9 pg/mL at first progression, 8.2 pg/mL at relapse, and 28.8 pg/ml in fludarabine-resistant CLL patients. After ibrutinib treatment, we found a decrease of big ET1 in plasma from 10.8 to 3.9 pg/mL ($p<0.05$).

Conclusion. The present study demonstrates that ET1 signaling is involved in cell homing, BCR activation and pro-angiogenic profile of CLL cells. Our results also envision the possibility to interfere with ET receptors activity using macitentan as a possible novel therapeutic strategy for CLL patients.

P029

IBRUTINIB MODIFIES THE FUNCTION OF NURSE-LIKE CELLS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. In lymphatic tissues, CLL cells establish intimate contact with accessory cells, such as nurse-like cells (NLCs). NLCs have a pivotal role in CLL clone maintenance and support CLL cell survival, proliferation and protection from drug-induced apoptosis. Ibrutinib is a potent inhibitor of BTK kinase, able to counteract the pro-survival effects in CLL cells provided by microenvironment leading to reduced retention and homing of CLL cells from tissue

compartments. Here, we investigated the biological effects mediated by ibrutinib on CLL-NLCs crosstalk.

Methods. CLL-PBMCs were cultured in complete medium for 12 days and then treated with 1 μM ibrutinib. NLCs were exposed to ibrutinib assessing cell surface markers, gene expression and functional properties. IL-10 production was tested by real time PCR and CSA in NLCs. CD19+ CLL cells viability and analysis of signaling pathways in presence of IL-10 stimulation were evaluated after treatment with ibrutinib.

Results. Ibrutinib targets BTK expressed by NLCs modifying their phenotype and function. We first observed that after ibrutinib treatment the viability of NLCs was not affected and the morphology of NLCs was preserved. Then, we investigated if ibrutinib exerts a direct effect on NLCs modulating specific markers typical of M1 and M2 polarization. In particular data show that ibrutinib acts on NLCs inducing a strong expression of genes involved in M2 polarization such as CD163 ($p<0.01$), IL-10 ($p<0.05$), MRC1/CD206 ($p<0.05$) and CCL18 ($p<0.05$), concurrently decreasing the expression of M1 polarization genes as IL1 ($p<0.01$), TNFα ($p<0.01$) and IL2 ($p<0.01$) compared to untreated control. Inside tissue microenvironments, NLCs establish a bidirectional cross-talk with CLL cells, allowing their protection from spontaneous and drug-induced apoptosis. We exposed both CLL cells and NLCs to ibrutinib. Ibrutinib does not completely hamper the protective effect mediated by NLCs on leukemic cells when both CLL and NLCs are exposed to the drug ($p<0.05$ at 24h, 48h and 72h). Among the plethora of factors potentially involved in CLL desensitization to ibrutinib effect, we focused our attention on IL-10 and we demonstrated that treatment with ibrutinib for 24h improves NLCs secretion of IL-10. CD19+ CLL cells stimulated with IL-10 in a dose escalation experiment (from 0.1 ng/mL to 100 ng/mL) show good protection from apoptosis at different doses at different time-points. We then analyzed the ability of ibrutinib to abrogate the pro-survival signal induced by IL-10 stimulation (1 ng/ml) in CD19+ CLL cells after 24-48h of culture. Ibrutinib does not completely antagonize the ability of IL-10 to protect CLL cells from apoptosis and to activate pSTAT3 and pERK signaling pathways.

Conclusion.: Our results provide new insights into the mechanism of action of ibrutinib that further miseducates NLCs, suggesting that this drug not only has an effect on the CLL clone but also extensively influences the cellular components of the CLL microenvironment.

P030

CHARACTERIZATION AND PROGNOSTIC RELEVANCE OF CIRCULATING MICROVESICLES IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: Given the relatively quiescent nature of chronic lymphocytic leukemia (CLL) and its clinical and biological heterogeneity, the identification of new prognostic biomarkers is a relevant issue in this lympho-proliferative disorder. Literature data have demonstrated that microvesicles (MV) deriving from CLL cells may affect the surrounding microenvironments, playing a key role on survival of neoplastic clone. We investigated phenotype and number of circulating MV in CLL patients in order to test their possible prognostic significance.

Patients and methods: Serum samples of 131 newly diagnosed CLL were retrospectively analyzed. MV were isolated by ultracentrifugation, counted and phenotyped by cytofluorimetric analysis, and finally correlated with the main clinical and biological disease's features.

Results: The absolute number of serum MV of CLL patients was significantly higher (1.104/μl± 846) with respect to healthy subjects (304/μl± 170) ($p<0.001$) (Figure1A). Moreover, by stratifying CLL patients for Rai staging system, we found that patients with advanced clinical stages (III-IV) had a significantly higher number of MV (3.967/μl± 7.390) with respect to patients with Rai stages 0 (1.272/μl± 955) and I-II (1.121/μl± 1.683) ($p<0.01$) (Figure 1B). In addition, B-cell derived MV, in particular CD37+ MV, significantly correlated with high tumor burden ($p<0.05$). Finally, absolute MV number cut-off selected by ROC analysis for time to treatment (TTT) and for overall survival (OS) distinguished Rai stage 0 patients with shorter TTT from those with more stable disease (median 75 months vs not reached, $P<0.01$), as well as, in the entire cohort, two groups of patients with different OS (median 127 months vs not reached, $P=0.02$), respectively (Fig.1 C and D). At multivariate analysis, serum MV independently predicted for OS, along with Rai stage.

Conclusions: Circulating MV represent a new potential prognostic biomarker in CLL.

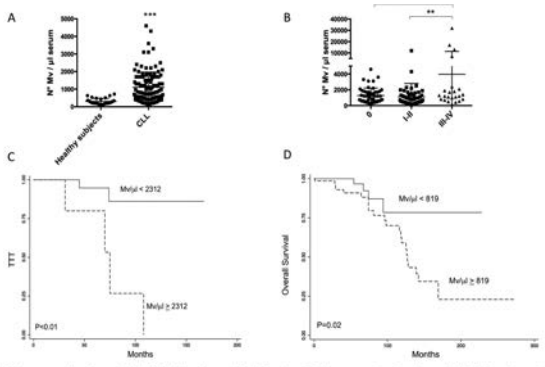

Figure1: (A) Mean concentration of serum MV in 28 healthy subjects vs 131 CLL patients. **(B)** Mean concentration of total serum MV in CLL patients stratified by Rai stage. **(C)** Kaplan-Meier curves obtained in 46 Rai 0 CLL patients by comparing TTT of patients with higher number of MV (MV≥2.312) with respect to patients with lower number of MV (MV<2.312) (log-rank test - *P*<0.01). **(D)** Kaplan-Meier curves obtained in 131 CLL by comparing OS of CLL patients with higher number of MV (MV≥819) with respect to patients with lower number of MV (MV<819) (log-rank test - *P*=0.02). Statistically significant results are indicated by asterisks: *P<0.05, **P<0.01.

P031

IN VITRO MODULATION OF PROGRAMMED DEATH-1 (PD1) RECEPTOR AND ITS LIGANDS PDL1 AND PDL2 PROTEINS IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) BY THE TUMOR MICROENVIRONMENT (TME)

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Introduction PD1 and its ligands (PDL1/2) form a dominant

immune checkpoint pathway operative in the TME and is activated in tumor cells to evade immune attack. PD1-expressing T-cells also highly express the CXCR4 receptor and its ligand SDF1a in the germinal centre of lymph nodes of CLL pts, where the axis is highly responsive to SDF1a. We report mRNA expression of PD1 and PDL1/2 in early-stage CLL samples (clinicaltrials.gov: NCT00917549); baseline protein expression and changes induced by exposure to autologous activated T-cells (AAT); and the effects of ibrutinib (IB), a BTK inhibitor, on this ligand/receptor axis in AAT co-culture. Methods GEP analysis was performed on purified B-cells (N=211). PD1, PDL1/2 expression (N=18) was evaluated in B and T cells by CFM (BD Biosciences). CLL/AAT cells, prepared using Dynabeads Human T-Activator CD3/CD28/IL2 (Thermofisher), were visually inspected for cluster formation (CF). Co-cultures were exposed to IB (1/10μm) for 24/48h either before or after CLL/AAT-CF. For migration assays, the chemoattractant SDF1a was used in presence/absence of IB (1/10 μM); analogous experiments were performed following co-culture of CLL and AAT. Results GEP analysis showed higher PD1, PDL1/2 expression in CLL cells vs normal B-lymphocytes subsets. A correlation was observed between PDL1 expression and mutated IGVH (p<.001), while there were no differences in expression of PD1 or its ligands with cytogenetic markers. Cases with either PDL1≥median values or PDL2≤median values had a significantly longer TTFT. ATT co-cultured with CLL upregulated expression of PD1 and its ligands, particularly in PDL1, compared to baseline (%PD1 17.5 vs 41.8, p<.0001; %PDL1 0.9 vs 19.6, p<.0001; %PDL2 7.0 vs 20.7, p=.0006); and CD8+PD1+ cells and CD8+PDL1 upregulation (%PD1 14.4 vs 25.4, p=.006; %PDL1 2.1 vs 12.9, p=.04) occurred in similar conditions; no changes were observed for CD8+PDL2 (4.1 vs 5.2, p=NS). CLL-AAT co-culture experiments with/without IB treatment 1/10μM IB for 24/48h showed a dose/time-dependent inhibition of CF by IB. There was a significant decrease in PD1 and PDL1, after 24h and notably after 48h of culture of CLL and CD8+T-cells. Inhibition of PDL2 protein in CLL cells was observed after 24h of 1nM IB, with modest changes observed for CD8+T-cells. Experiments after CF indicated no relevant changes in PD1, PDL1/2 expression in either B or T-cells induced by IB. CLL migration assays showed that SDF1a migration was reduced dose-dependently by IB, together with a reduction in actin polymerization and BTK-phosphorylation. Analogous migration assays following CLL/AAT co-culture showed a moderate increase in migration both in presence of SDF1a and interestingly, also by IB treatment. Conclusion TME-derived signals regulate expression of PD1 and PDL1/PDL2 in CLL and T-cells. IB inhibits SDF1acell migration only when not influenced by the TME. IB inhibits TME signaling and up-regulates PD1/PDL1/PDL2 expression suggesting a rationale for target therapy.

P032

NEXT GENERATION SEQUENCING (NGS) ION TORRENT AMPLISEQ-TM TECHNOLOGY FOR THE IDENTIFICATION OF TP53 MUTATIONS - SCREENING OF 59 CASES OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Background: We describe the application of a NGS panel based on Ion AmpliSeqTM technology designed to analyze the TP53 sequence in a single workflow.

Methods: Purified B-cells from 59 CLL samples were obtained from patients (pts) visiting our outpatient clinic. Library preparation and sequencing were performed on the Ion TorrentTM-PGM platform (Thermofisher) according to the manufacturer's protocols. For each run, 10 pooled samples were loaded on a 316 chip and sequenced (flow rate 500x). Data processing, filtering and base calling were performed using the Ion Torrent server v5. Based on Variant Caller Parameters we accepted all variants with an allele frequency (VAF)>3% with high quality or variant coverage >100, rejected variants had VAF<3% and low quality. All genomic sequences were compared to the IARC TP53 database.

Results: ION PGM sequencing run data showed a high coverage in 90% of the sequenced amplicons with average coverage uniformity of 89%, an average mean depth of 12253 (range 8112–17960), and 97% of mapped reads on target. Overall, we identified 129 variants in 59 pts in the TP53 full sequence. Overall, of the 59 pts analyzed 33 (57.6%) presented variants in TP53 exonic regions. Moreover, 10 pts with no detectable alterations, 10 pts with intronic mutations, 1 pt with mutation in the, or 5 pts having neutral alterations were considered WT; overall 26 TP53-WT pts were identified. 10/55 (18%) clonal mutations, defined as VAF>20% were identified and 45/55 (82%) presented only subclonal variations (VAF) 3-20%; 2 pts presented both clonal and subclonal variations. Of the 55 exon variants identified, the following effects at the protein level: 49 deleterious, 3 frameshift, 1 stop/loss, and 2 unclassified mutations and were indicated as alterations likely producing nonfunctional protein according to in silico analysis. The mutation in the 3'UTR could potentially affect regulation of TP53 gene expression by targeting miRNA. Correlation with FISH analysis showed that of the 4 pts with del17p, 3 pts presented a clonal TP53 mutation (with VAF range 22.5–94.7%) in the remaining allele. The 4th patient also presented a subclonal neutral mutation with a VAF of 3.1%. Of note this was the only del17p patient alive in this cohort.

Conclusions: The ION PGM TP53 panel offers an easy to use platform for the evaluation of small clones of TP53 mutations. A high percentage (82%) of mutations were detected as TP53-mutant subclones having a VAF<20%, that would likely be considered WT by Sanger sequencing, demonstrating the highly sensitive of the technique. The presence of subclonal mutations could anticipate the development of a chemo-refractory phenotype among CLL pts requiring treatment. We are currently evaluating TP53 clonal alterations identified by NGS screening in these CLL pts prospectively.

P033

PREDICTIVE POWER ON PROGRESSION FREE SURVIVAL (PFS) OF TP53 MUTATIONAL STATUS BY NEXT GENERATION SEQUENCING (NGS) ION TORRENT AMPLISEQ-TM TECHNOLOGY IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) PATIENTS TREATED WITH THE COMBINATION SCHEDULE OF BENDAMUSTINE AND RITUXIMAB (BR) AS FIRST-LINE – SCREENING OF 39 CASES

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Introduction: We recently reported a series of 279 CLL patients (pts) treated with the combination schedule of bendamustine and rituximab (BR) as 1st-line therapy. Of the 279 pts, 43 were analyzed

using Ion AmpliSeqTM technology for TP53 mutations. Herein, using a highly sensitive ultra-deep-NGS approach capable of detecting few mutated cells, we tested the predictive power of TP53 mutational status on response to therapy and progression free survival (PFS).

Methods: Purified CLL samples were obtained from 43 pts before BR therapy start. Library preparation and sequencing were performed on the Ion TorrentTM-PGM platform (Thermofisher) according to the manufacturer's protocol. Ten pooled samples/run were loaded on a 316 chip and sequenced (flow rate 500x). Data processing, filtering and base calling were performed using the Ion Torrent server v5. Based on Variant Caller Parameters we accepted all variants with allele frequency (VAF)>3% with high quality or variant coverage>100. All genomic sequences were compared to the IARC TP53 database. Cases presented variants along the exonic regions of the TP53 sequence were considered TP53 mutated (Mut), while cases with no detectable alterations, intronic mutations, mutation in the 3'UTR, and with neutral alterations were considered TP53 wild-type (WT).

Results: Of the 43 pts, 28 were males (65.1%), 17 had Binet stage C; median age was 70 years (range, 43-86 years). FISH analysis showed 4 (9.3%) had a del17p. Overall, 24 cases (55.8%) were TP53-Mut, while 19 (44.2%) were WT. Of the 24 TP53 mutated cases, 19 showed a TP53-Mut subclone with a VAF<20%, while 5 had clones with a VAF≥20%. Three of the 4 cases with del17p presented a clonal TP53 mutation (VAF range 22.5-94.7%) in the remaining allele, while the 4th pts also presented a subclonal neutral mutation with a VAF of 3.1%. The overall response rate (ORR) of the entire cohort was 86.1% with a complete response (CR) rate of 34.9%. No significant correlation between TP53 mutational status and either ORR or quality of response was observed (TP53-WT cases vs TP53-Mut cases; ORR: 100% vs 90%; P=.157; CR: 42.1% vs 35%; P=.168). Instead, TP53-Mut cases showed significantly shorter PFS than those with TP53-WT (TP53-WT cases vs TP53-Mut cases; 2-yr PFS: 90% vs 67.6; P=.036). Using a TP53 VAF cut-off of 20%, 38 pts were considered negative and 5 positive. Pts with a VAF<20% for TP53-Mut showed a significantly longer PFS than those with a TP53-Mut with VAF≥20% (2-yr PFS: 89.7% vs 0%; P<.0001). CONCLUSIONS: The ION PGM TP53 panel is an easy-to-use platform for the evaluation of small clones of TP53 mutations. This methodology allows detection of a high percentage (79.2%) of TP53 mutations having a VAF<20% likely to be considered WT by Sanger sequencing (SS), demonstrating the highly sensitive of the technique. The presence of TP53 mutations by NGS identifies CLL pts with a short time to progression after first-line BR therapy. Nonetheless, TP53 mutated pts with VAF≥20%, also detectable by SS, showed worse outcome.

P034

REGULATION OF HIF-1α IN TP53 DISRUPTED CHRONIC LYMPHOCYTIC LEUKEMIA CELLS AND ITS POTENTIAL ROLE AS A THERAPEUTIC TARGET

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Treatment of high-risk chronic lymphocytic leukemia (CLL) patients remains an unmet clinical need. Disease aggressiveness can be ascribed to intrinsic features of the tumor cells, such as TP53 disruption, and to crucial interactions of CLL cells with the tumor microenvironment. The transcription factor HIF-1α is critically involved in the regulation of genes implicated in tumor progression and modulates CLL cells interactions with stromal cells (SC). Its expression and transcriptional activity depend on genetic alterations of tumor suppressor genes (i.e TP53), oxygen deprivation and soluble factors. CLL cells express HIF-1α even in normoxia, and its expression is rapidly elevated during hypoxia. We have already reported that SC upregulate HIF-1α activity in CLL cells via activation of Akt, and Ras/ERK1-2 and RhoA/RhoA kinase signaling pathways.

The aim of this study was to understand the HIF-1α regulatory pathways and to evaluate the ability of HIF-1α inhibition to potentiate fludarabine cytotoxicity in TP53 disrupted (TP53^dis) CLL cells.

CLL cells were cultured alone or with the M2-10B4 SC line under normoxic or hypoxic conditions. Cell cultures were exposed to fludarabine (10 μM), simvastatin (Sim, 1 μM), PD89059 (PD, 10 μM) or BAY87-2243 (BAY, 1 μM). Ras and RhoA activities were evaluated by pull-down assay and ELISA, respectively. ERK1-2 and HIF-1α were evaluated by Western Blot. RhoA kinase, Akt and HIF-1α activities were measured with specific immunoassay. Glycolitic activity was measured by a 6-14C-glucose radiometabolic assay. Cell viability was analyzed by Annexin-V/propidium Iodide immunostaining and flow cytometry. Patients with TP53 mutation or 17p deletion were considered TP53^dis.

TP53^dis CLL cells had a constitutive upregulation of HIF-1α protein and a significantly higher transcriptional activity compared to TP53^wt CLL cells. As a result, TP53^dis CLL cells overexpressed the glycolytic HIF-1α target genes ENO1 and PGK, and had a more active glycolysis than TP53^wt cells. Oxygen deprivation further increased HIF-1α expression and activity in TP53^dis CLL cells cultured for 48 hours under hypoxic conditions. Similarly, co-culture of TP53^dis CLL cells with SC upregulated HIF-1α expression and activity via activation of Akt, and Ras/ERK1-2 and RhoA/RhoA kinase signaling pathways. In TP53^wt CLL cells, Sim and PD significantly counteracted the SC-mediated protection from spontaneous and fludarabine-induced apoptosis, but this effect was not observed in TP53^dis CLL cells. Conversely, the HIF-1α specific inhibition by BAY potentiated the cytotoxicity of fludarabine on TP53^dis CLL cells, also counteracting the protective effect exerted by SC.

Our data demonstrate that TP53^dis CLL cells constitutively overexpress HIF-1α, which is further up-regulated by hypoxia and SC. HIF-1α targeted inhibition is a potential strategy to circumvent constitutive and SC-induced fludarabine resistance of TP53^dis CLL cells.

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P035

VORINOSTAT IMPROVES THE ANTITUMOR EFFICACY OF RUXOLITINIB IN HEMATOLOGICAL CELL LINES

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Background and Objectives. Jak-STAT pathway is a critical signaling cascade for the transduction of extracellular signals to the nucleus and regulation of gene expression. Over activation of the Jak/STAT pathway has been reported in subsets of patients of hematologic malignancies. Ruxolitinib, a first in class Jak1/2-specific inhibitor, has been approved for the treatment of myelofibrosis. It achieves symptomatic improvement in patients but does not eliminate the neoplastic clone, and combinations of Ruxolitinib with other compounds are under investigation. Its potential role in other hematologic malignancies still needs to be fully researched. In this study, we analyzed the effects of Ruxolitinib alone and in combination with Vorinostat in ten different hematologic malignancies cell lines: multiple myeloma, Hodgkin lymphoma, non Hodgkin lymphoma and Chronic lymphocytic leukemia. Methods. Cell lines underwent a screening test in order to identify their sensibility to single agents. Cell lines alone, and co-cultures with bone marrow stromal cells were treated with increasing concentrations of Ruxolitinib (0,05 nM to 2 nM) and Vorinostat (0,5uM to 20uM) for 24 and 48 hours and the IC50 values were analysed by MTT assay. The combination index was calculated by Chou-Talalay method. Apoptosis and cell cycle were studied by flow cytometry. The effect of the treatment on phosphorylation of protein kinases was evaluated by western blot analysis. ROS generation was studied using the fluorophore H2DCFDA. Results. The preliminary results show that Ruxolitinib and Vorinostat as single agents had a cytotoxic effect at 24 h with cytotoxicity against all cell lines after 48h of treatment. However the combination of low doses of both drugs showed a clear synergistic interaction just after 24h of treatment. In our study, Ruxolitinib and Vorinostat alone exerted only moderate effects on ROS generation, whereas combined treatment resulted in a substantial activation of the mitochondrial pathway by the accumulation of ROS. Apoptosis induced by single drugs was mediated by activation of caspase 3 and -9, and moderate activation of caspase 8 which expression was increased after the combination. Concordant with the enhanced apoptosis, we observed an effect on the expression of apoptosis-regulating proteins, with up-regulation of Bax and BAD and down-regulation of Bcl-2 and Mcl-1 . Ruxolitinib and Vorinostat as single agents resulted in a dose dependent down regulation of Cyclin D1 at 48h and a significant decrease in the S-Phase entry of the cell cycle. The same effects were evident at an earlier time point of treatment with the combination of Ruxolitinib and Vorinostat. These very preliminary results *in vitro* suggest that the combination of Ruxolitinib and Vorinostat is of potential value for the treatment of haematological diseases and further research are ongoing.

P036

ROLE OF BENDAMUSTINE-BORTEZOMIB-DEXAMETHASONE (BVD) IN THE MANAGEMENT OF RELAPSED AND REFRACTORY MULTIPLE MYELOMA: A REAL-LIFE EXPERIENCE

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Bendamustine is a bifunctional alkylating agent, with low toxicity, proved to be effective in relapsed, refractory and in new diagnosed Multiple Myeloma (MM). It has been evaluated efficacy and tolerance of Bendamustine, in combination with bortezomib-dexamethasone (BVD) in patients with relapsed and refractory MM (rrMM), whose prognosis is particularly severe. A regional retrospective real-life analysis of patients with rrMM who had been treated with BVD as salvage therapy has been performed. 47 patients (25 M/22 F), with rrMM, median age at diagnosis 58.4 years (range 36-82), median age at start of treatment 61.3 years (r.37-83), treated with several lines of treatments (median 6, r. 2-11), every refractory to all the drugs previously received (also Bortezomib), received BVD (Bendamustine 90 mg/sqm days 1,2; Bortezomib 1.3 mg/sqm days 1,4,8,11, Dexamethasone 20 mg days 1,2,4,5,8,9,11,12, Pegfilgrastim day +4) every 28 days, until progression. ISS was equally distributed, and cytogenetic was evaluable in 9 patients, and in particular one del13q and one t(11;14). All the patients had previously been treated with schedule containing bortezomib and IMIDs, 90% of them with melphalan, 77% with cyclophosphamide, 34% with antracyclines and 30% had also received radiotherapy. 58% of them had undergone at least to a single autologous SCT. All patients were relapsed and refractory to last therapies received before BVD. Bendamustine was well tolerated, with grade 3 transfusion-dependent anemia in 29% of patients, and 41% grade 3 neutropenia (no ospedalization was required, no septic shock was observed). No severe extrahematologic toxicity was observed, only grade 1 gastrointestinal side effect (nausea), treated by common antiemetic drugs. According to IMWG, after a median follow-up of 9 months (r.2-36), ORR was 57% (27/47 : 2 CR, 3 VGPR, 14 PR, 8 MR) with 8 PD and 12 patients in SD, which can be considered as an impressive result in this subset of rrMM patients. In particular, for 6 patients, BVD was, after having achieved a PR, a bridge to second autologous SCT, and for one patient a bridge to allogenic SCT. Median time to response was 1.3 months (r.1-3), median OS from diagnosis was 61.4 months (range 6-151), median OS from start of Bendamustine was 9.3 months (range 2-36).

BVD has shown significant efficacy (ORR 57%) in a particularly severe setting of patients, relapsed and refractory to all available therapeutic resources, and in particular cases it could be considered as a bridge to a second autologous or allogenic BMT.

P037

PRIMARY HEPATIC NON HODGKIN LYMPHOMA: HIGH PREVALENCE OF HEPATITIS C VIRUS INFECTION AND EFFICACY OF THE CONVENTIONAL SIX COURSES OF ANTHRACYCLINE-BASED CHEMOTHERAPY

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Primary Hepatic non-Hodgkin's Lymphoma (PH-NHL) is a form of cancer characterized by the exclusive involvement of liver at the moment of the diagnosis. Even if its occurrence is rare, PH-NHL should enter in the differential diagnosis of every space-occupying liver lesion. Only very small series of such lymphoma entity have been reported in literature, and among them a non-fortuitous association with Hepatitis C Virus (HCV) infection has been shown. The prognosis is believed to be dismal, with early recurrence and short survival. In this retrospective study, we assessed the longest series of PH-NHLs until now reported, in order to perform a more in-depth analysis of the impact of HCV infection and antineoplastic therapeutical interventions on patients'outcome. Among a population of 600 patients with NHL seen at our institution between 1990 and 2014, 14 (about 2%) received a biopsy-proven diagnosis of PH-NHL and were included in this study. Histological subtypes were DLBC (n : 6), follicular (n : 4), MALT (n: 1), small lymphocytic

(n : 2), and peripheral T-cell lymphoma (n : 1). The rate of prevalence of HCV-infection among PH-NHL was 78.5% (11/14), and of them (7/11, 63.6%) had viral liver disease (3 cyrrhosis, 4 chronic active hepatitis). Standard schedules (regarding dose-dense and dose-intensity approach) of six courses of anthracycline-based chemotherapy or rituximab-based immune-chemotherapy, or radical surgery were the mainstay of treatment. Complete remission rates was 100%. At time, 13/14 (93%) patients are alive with a median disease free survival of 150.5 months (range 36-223) and a median overall survival of 164 months (r. 40-228). In the rituximab-based group, 50% of patients (3/6) had a recurrence of HCV infection with deterioration of liver function tests and/or histology, whereas none of the patients in the anthracycline subgroup had this reactivation. Our study confirms the rarity of PH-NHL, and shows a very high prevalence rate of HCV infected patients and favourable lymphoma outcome with conventional anthracycline-based chemotherapy. The toxic side effects should lead to consider the role of rituximab and/or aggressive surgery in this particular subset of patients.

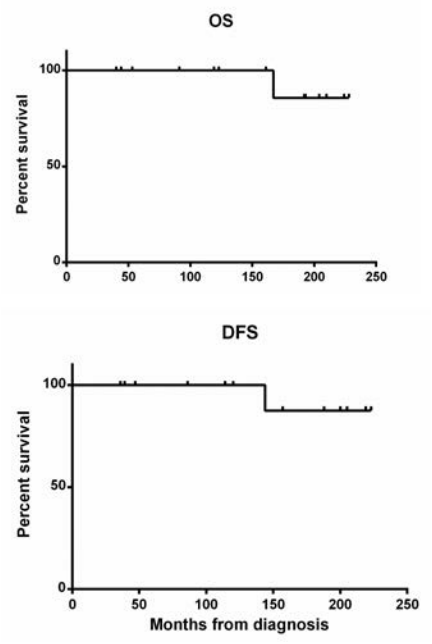

Figure 1.

P038

IGD MULTIPLE MYELOMA IN THE "NOVEL AGENTS ERA": A DESCRIPTIVE SINGLE-CENTER REPORT OF 5 CASES. CLINICAL PROFILE, SURVIVAL AND RESPONSE TO THERAPY

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Introduction: Immunoglobulin D multiple myeloma (IgD MM) accounts for almost 2% of all myeloma cases. It is associated with osteolytic lesions, extramedullary involvement, amyloidosis, renal failure, hypercalcemia. It has been reported with a poorer outcome compared with other subtypes, with an aggressive course, but after introduction of novel agents clinical course and prognosis has improved. Moreover data about IgD myeloma response rate are lacking owing to rarity of this condition. In this report we want to share our experience of IgD MM patients.

Methods: We report five IgD MM patients, four female and one male, diagnosed between 2001 and 2015 at our institution. They received chemotherapy with novel agents alone or followed by autologous hematopoietic stem cell transplantation (ASCT). Three of them performed ASCT; two were treated with new drugs and chemotherapy, without transplant, according to old age.

Results: Median age was 58 years (range 39-75). Four patients were diagnosed IgDlamda MM (80%), while one had IgD kappa (Figure 1). Clinical frontline characteristics are: for most of patients (4 cases, 80%) extramedullary involvement , severe bone disease (100%), hypercalcemia (60%) and fever (60%). LDH levels at diagnosis were high in all patients. Patient characteristics are summarized on Table 1. Only one patient received VAD regimen before "new drugs era" as first line treatment, while the rest was treated with bortezomib containing regimen. Median overall survival was 62 months (range 15-168). All patients reported have obtained a complete remission and are currently being monitored in followup at our centre. Our series of patients has presented an aggressive clinical picture, with onset of advance disease. Two patients described reported the same chromosomal aberration (trisomy of chromosome 5 and 9) never shown before in this kind of myeloma. One patient presented trisomy of chromosome 1 that disappeared after treatment. Our patient population reported is very heterogeneous because we show prognosis improved of 2 elderly patients not elegible for ASCT as well as 3 young patients undergoing transplant procedure. Nevethless all of them have obtained a complete remission and preserved over time.

Conclusions: For the past few months we heard debates between MM experts about potential curability of MM. Probably it might be feasible even for patients with IgD MM, with the introduction of new drugs and transplant procedure. In this report we showed that IgD myeloma patients responded better to current new treatment and had a higher CR rate expecially after ASCT. Despite the heterogeneity of the population, therapeutic use of the novel drugs both in first line therapy and relapsing disease achieved an improvement in overall survival. These results were however obtained in few patients and additional studies, i.e. an international collaborative trial, involving a larger number of patients are warranted, in order to confirm our encouraging data and better explain the pathogenesis and clinical behavior of this rare and very interesting disease.

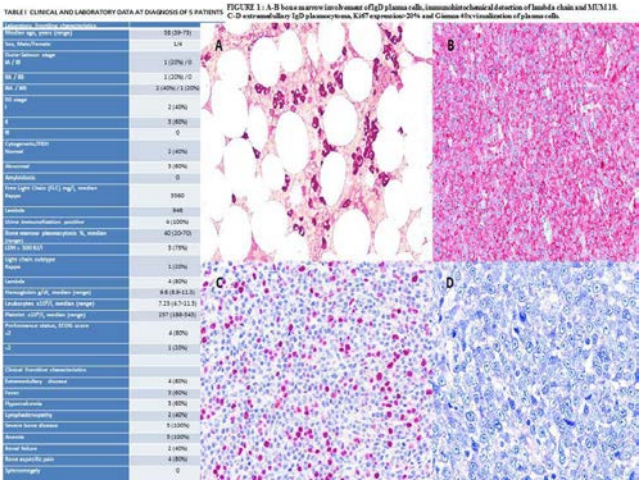

Figure 1.

P039

ABNORMAL SERUM FLC RATIO AND IMMUNOPARESIS IN PATIENTS WITH MGUS: DIFFERENCES BETWEEN IGA AND IGG MGUS

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Background: Monoclonal gammopathy of undetermined significance (MGUS) is the most common disease among the plasma cell dyscrasias. It occurs in 3% of people older than 50 years and up to 10% in those older than 70; it is associated with a 1%/year risk of progression to Multiple Myeloma (MM). Free Lights Chains ratio, immunoparesis, type and amount of the monoclonal component are considered parameters predictive of progression. In patients (pts) with MGUS there is a significant relationship between abnormal k/l ratio and immunoparesis. When k/l ratio is altered it is also common to find a more pronounced immunoparesis, often with simultaneous reduction of two classes of immunoglobulins. Previous studies reported IgA MGUS is at higher risk of progression than IgG MGUS. We indirectly confirmed these data: in our cohort of patients, IgA gammopathies are 13% of MGUS cases and 20% of Multiple Myeloma cases.

Patients and methods: We evaluated FLC ratio and immunoparesis in 22 pts with IgA MGUS and in 144 with IgG MGUS (Binding Site method).

Results: k/l ratio was altered more frequently in IgA MGUS pts than in IgG, although the difference is not statistically significant (68.1% vs 49.3 P = 0.094318). The percentage of pts with monoclonal light chain above the upper limit was significantly higher in IgA MGUS than in IgG MGUS (86.3% vs 62.3% P = 0.027224). The occurrence of immunoparesis was significantly higher in IgA than in IgG MGUS. (63.6% vs 31.9% p=0.004894). The percentage of pts with reduction of the polyclonal light chain was not statistically different in the two types of MGUS (68.1% vs 64.2% P= 0.716331). Pts with IgA MGUS had more frequently the simoultaneous presence of immunoparesis and altered k/l ratio. (76.9% vs 35.1% P = 0.005146)

Conclusions: Our data underline a different behaviour of IgA and IgG MGUS. Most interestingly, in pts with IgA MGUS an association between immunoparesis and altered k/l ratio is more frequent than in pts with IgG MGUS. We suppose that there may be a relationship between this biological aspect and the clinical aggressiveness of IgA MGUS.

P040

EPIGENETIC SILENCING BY METHYLATION OF SIRT1, KLF4, DAPK1 AND SPG20 IN NON-HODGKIN LYMPHOMAS

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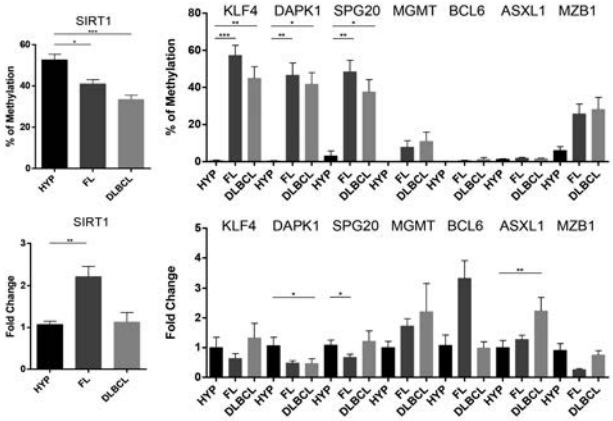
Introduction: Non-Hodgkin lymphomas (NHL) represent the vast majority of all diagnosed lymphomas. Among them, diffuse large-B cell lymphomas (DLBCL) and follicular lymphomas (FL) are the most represented subtypes. Epigenome of lymphomas presents heterogeneity and epigenetic regulators or chromatin modifiers can be considered promising targets. Epigenetic regulation of gene expression plays a center role in cancer initiation and progression. DNA methylation represents one of the major epigenetic mechanisms of gene regulation. SIRT1 is a class III lysine-deacetylase playing several functions and considered to be a context-dependent tumor promoter.

Methods: Lymph nodes were collected within the Tissue Bank project. Transcription levels were assessed by quantitative RT-PCR. Methylation were performed by pyrosequencing and EpiTect Methyl II PCR. Data were analysed with a Prism 5.0 software (Graph Pad).

Results: SIRT1 is preferentially expressed in the B lymphocytes of the germinal center of the follicle. SIRT1 mRNA levels are higher in FL than follicular hyperplasias and DLBCL and its expression positively correlates with BCL6, one of the master regulator of germinal center in the follicle. SIRT1 promoter shows a methylation decrease in the order: follicular hyperplasia - FL - DLBCL, while BCL6 promoter does not show methylation differences between the samples. We also investigated promoter methylation and gene expression of 7 different genes (KLF4, DAPK1, SPG20, MZB1, MGMT, LMO2 and ASXL1) potentially relevant for lymphoproliferative malignancies. KLF4, DAPK1 and SPG20 show a methylation increase in FL and DLBCL compared to follicular hyperplasias. Gene expression of DAPK1 and SPG20 anti-correlates with their degree of methylation, while KLF4 mRNA levels are not changing among samples. To elucidate the functional role of SIRT1, we evaluated SIRT1 expression and activity on two DLBCL cell lines derived from GC- or ABC-like subtypes. Notably, these cell lines feature a good anti-correlation between gene expression and promoter methylation on KLF4, BCL6, DAPK1, MGMT, SPG20, ASXL1 and MZB1. In this respect, we want to determine whether the disruption of SIRT1 function could determine mechanisms of reactivation coinciding with any changes in the DNA methylation status.

Conclusions: SIRT1 emerged recently as a player of lymphoma cells apoptosis and cell cycle arrest. Its over-expression or enzymatic activation results in aberrant histone deacetylation and DNA methylation. Epigenetic changes in SIRT1 methylation inversely correlate with NHL aggressiveness, while KLF4, DAPK1 and SPG20 show a methylation increase that correlates with tumor aggressiveness. Thus, different patterns of methylation correlate with the clinical and prognostic parameters and histone deacetylation and DNA methylation acts in synergistic way to control gene expression.



Quantitative methylation and gene expression analysis. Top. Quantitative methylation on CpG sites of SIRT1, KLF4, DAPK1, SPG20, MGMT, BCL6, ASXL1 and MZB1. Bottom. Gene expression by RTqPCR of the genes whose methylation is significantly different in the B lymphocytes from the three types of lymph nodes. HYP: Follicular hyperplasia (black), FL: follicular lymphoma (grey), DLBCL: diffuse large B cell lymphoma (light grey).

Figure 1.

P041

STUDY OF GENE POLYMORPHISMS AS PREDICTORS OF TREATMENT EFFICACY AND TOXICITY IN PATIENTS WITH INDOLENT NON-HODGKIN LYMPHOMAS RECEIVING BENDAMUSTINE AND RITUXIMAB

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Background: Indolent non-Hodgkin lymphomas (iNHL) have a heterogeneous behavior, impacted by biological and clinical parameters. Bendamustine is widely used in association with rituximab to treat iNHL. The variability in treatment efficacy and toxicity could be related to genetic factors of the host, such as germline single nucleotide gene polymorphisms (SNPs) in genes that affect drug disposition, pharmacodynamics and the components of reactive microenvironment. Gene polymorphisms have been showed to influence NHL outcome. Deregulation of some components of the immune system (for example cytokines network) and angiogenesis could play a role for tumor growth and survival. Genetic variants in immune and inflammatory response genes (such as the ones coding for IL-10 and IL-6) and in angiogenic factors (such as VEGF) could affect clinical outcome as well as side effects.

Aim: To demonstrate a correlation between SNPs and treatment outcome in iNHL patients receiving bendamustine and rituximab.

Methods: This study includes the collection of efficacy and toxicity data and a biological part with DNA extraction and genetic analyses. We will investigate some SNPs that have already been associated with treatment efficacy and toxicity in NHL patients. Genomic DNA was extracted using silica membranes (Qiagen DSP DNA Blood Kit). All samples were genotyped for the IL-2 (rs2069762), IL-10 (rs1800890, rs10494879), VEGFA (rs3025039), IL-8 (rs4073), CFH (rs1065489) and MTHFR (rs1801131) SNPs by allelic discrimination assays using TaqMan SNP Genotyping Assays (Applied Biosystem) containing primers forward and reverse and allele specific MGB (Minor Groove Binder) probes. SNPs assays were executed on a Rotor Gene 3000 platform system (Corbette, Explera) and the analysis of genotyping were performed using the Rotor Gene Software.

Results: We have enrolled 40 consecutive iNHL patients and herein we report a pivotal analysis of the first 20 patients with a follow-up of at least 6 months that received rituximab 375 mg/m^2 and bendamustine 90 mg/m^2 every 28 days both as first-line treatment (14/20) and as ≥ 2nd line regimen (6/20). Overall response rate was 100% (CR rate 70%). Treatment toxicity included grade 3-4 neutropenia (10/20 patients), infections (8/20 patients; 1/8 grade ≥3), skin rash (10/20 patients; 1/10 grade ≥3). SNPs in IL-2, IL-8, MTHFR were observed in 10, 9 and 9 patients, respectively; while the other investigated genes were wild type for all patients. We showed a trend between the lack of CR and wild type allele for all these 3 genes (p=0.06). Moreover, we observed an association between SNP in IL-2 (rs2069762) and skin rash (p=0.02).

Summary/Conclusion: We confirm treatment efficacy and manageable toxicity of rituximab and bendamustine for iNHL patients; median follow-up is too short to report survival analyses. Preliminary results of our study suggest a possible role for cytokine SNPs in bendamustine-related toxicity, that needs to be confirmed in a larger cohort.

P042

VITAMIN D DEFICIENCY AND SUPPLEMENTATION IN DIFFUSE LARGE B CELL LYMPHOMA: IMPACT ON OUTCOME

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Data from the German RICOVER-60 study indicate that vitamin D deficiency is a risk factor in elderly patients (pts) with DLBCL treated with Rituximab-containing chemotherapy (R-CHOP) (Bittenbring et al, J Clin Oncol 2014). *In vitro* data suggest that vitamin D supplementation could enhance rituximab-mediated cytotoxicity. In a single-center study, we prospectively measured vitamin D levels in a cohort of 150 newly diagnosed pts with DLBCL from May 2013 to March 2016 who were candidates for Rituximab-containing chemotherapy. Pts with deficient/insufficient vitamin D levels were offered supplementation. Vitamin D levels were controlled during supplementation. Event Free Survival (EFS) was defined as time from diagnosis to relapse, disease progression or change of therapy for any reason or death. Vitamin D levels were considered deficient (<10 ng/mL) in 54 pts (36%), insufficient (10 to 30 ng/mL) in 78 pts (52%), and normal (>30 to 100 ng/mL) in 18 pts (12%). Looking at pts characteristics, there was no difference in vitamin D levels with sex (p=0.34), stage (p=0.9) or advanced IPI (p=0.46), while pts with poor performance status (ECOG > 2), had significantly lower vitamin D levels (p=0.005). Moreover, Vitamin D levels correlated negatively with Hb and albumin levels (Spearman's rank coefficient p=0.004 and p=0.0005, respectively). In addition, there was a significant seasonal variation with lowest vitamin D levels in the second trimester (p=0.003). As normalization of vitamin D levels have been shown to improve in-vitro rituximab-mediated cellular cytoxicity by NK cells, we implemented a substitution regimen to increase vitamin D levels early during treatment. We supplemented vitamin D (cholecalciferole) in a daily dose of 25000 U for a period that varied according to the initial vitamin D levels and subsequently continued maintenance with cholecalciferole 25000 U once a week. A second determination of Vitamin D levels after a median of 1.4 month in 78 pts showed a significant increase of Vitamin D levels from 12 ng/ml to 30 ng/mL (p=0.001). Supplementation resulted in normalization of Vitamin D levels in 38/78 pts (49%) No episodes of hypervitaminosis or hypercalcemia were observed. We analyzed the prognostic impact of vitamin D levels at diagnosis and after supplementation. Pts with vitamin D levels in the normal range either at diagnosis or due to supplementation had a better 2-year EFS when compared to pts with persistently deficient/insufficient vitamin D levels (89% versus 49%, p=0.005). Vitamin D levels at diagnosis before supplementation only showed a trend for impact on EFS (p=0.08). We conclude that Vitamin D deficiency is frequent in pts with DLBCL also in central Italy. Vitamin D supplementation results in improved vitamin D levels, and normalization of vitamin D levels associate with favourable outcome. These data suggest that outcome in DLBCL pts treated with rituximab-containing regimens may be improved by vitamin D supplementation.

Molecular Hematology

P043

SNP RS1333040C>T IN CHRONIC MYELOID LEUKEMIA PATIENTS: SINGLE CENTER EXPERIENCE

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Treatment with TK inhibitors (TKIs) has drastically changed the outcome of patients affected by CML,but the use of TKIs is associated with increased risk of cardiovascular (CV) events. The chromosomal locus 9p21 is a novel genetic marker for a variety of CV diseases. Single nucleotide polymorphisms (SNPs) on this chromosomal region have been recently associated with myocardial infarction, atherosclerosis of coronary arteries, ischemic stroke, and peripheral artery disease. In this study, we focused on SNP rs1333040C>T - which is one of the SNPs on chromosome 9p21 that has been more frequently and consistently associated with ischemic CV diseases in the medical literature - and evaluated the distribution of its three possible genotypes in a series of patients affected by CML and treated with TKIs. The goal was to use this prototypical SNP to evaluate a possible association between genetic factors and TKI-induced CV events.

Patients and Methods: We studied 93 subjects,median age was 52 years; 44 were males and 49 were females. All patients were treated with a median of 1 TKI (range 1-3), with imatinib being the drug most commonly used as first line therapy (76%). For all patients, the theoretical CV risk was calculated using the European Score Charts published by the European Society of Cardiology. The occurrence of CV events before and during treatment with TKIs was recorded for all patients. For the genetic studies, genomic DNA was isolated from peripheral blood and the rs1333040C>T genotypes were assessed by PCR-RFLP. Association of genotypes with the incidence of CV events was calculated using Pearson's œá2 test. A value of p<0.05 was considered statistically significant.

Patients characteristics	C/T subgroup	T/T subgroup
Sex (M/F)	24/30	19/20
Median age	51,5 years (range 18-84)	53 years (range)
Sokal risk		
low	30	17
Intermediate	12	13
high	12	8
Cardiovascular risk score	C/T subgroup	T/T subgroup
0-1	35	26
2-5	9	4
>5	11	11

Results: Among the 93 subjects enrolled in the study, the genotype distribution was: 39 (42%) TT, 44 (58%) CT, 0 (0%) CC. In group of subjects with the CT genotype, 3 (6.8%) presented CV events. Of these, two were transient ischemic attacks (TIA) occurred before the initiation of TKI therapy, in a two women with scores 5 and 2 at the CV risk tables, respectively. The third event was peripheral artery disease (PAD), in a woman with a score of 10, receiving nilotinib 800 mg/d as third line treatment. In the group of subjects with the TT genotype, 9 (23.0%) presented CV events. Of these, six were myocardial infarctions (MI) that occurred during TKI treatment. One patient presented a TIA and other two developed PAD during treatment. The difference in the incidence of CV events between subjects with the TT and the CT genotypes was statistically significant (p=0.02).

Discussion: The TT genotype of the rs1333040C>T polymorphism on chromosome 9p21 has been consistently associated with CV diseases in the general population. In this study, we evaluated patients affected by CML and treated with TKIs, in whom the incidence of CV diseases is particularly high. We found a higher incidence of CV events among subjects carrying the TT genotype. These results suggest a possible role of the 9p21 chromosomal region in the pathogenesis of CV events among CML subjects treated with TKIs.

P044

RELIABLE STANDARDIZED PCR METHOD FOR BCR/ABL TRANSCRIPT SCREENING: A COMPARISON BETWEEN BIOMED-1 PROTOCOL AND COMMERCIAL KITS

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Introduction: The molecular characterization of bcr/abl transcripts is a pivotal step in the diagnosis of Chronic Myeloid Leukemia (CML) and Ph+ Acute Lymphoblastic Leukemia (ALL). Unfortunately, this analysis is not available in every laboratory. In Italy a network of more than 50 standardized laboratories (LabNet) has been developed, performing bcr/abl analysis for nationwide hematologic units/labs. However, smaller labs could benefit of a rapid, in-house, certified method to perform the qualitative characterization of bcr/abl transcripts at diagnosis, thus reducing time, costs and shipment mistakes. The aim of this work is compare the specificity, reliability and time/cost-saving of the standardized BIOMED-1 protocol [1] for qualitative analysis of bcr/abl and two commercial kits. We tested also the association with 3 different reverse transcription (RT) methods.

Methods: Eight CML and three ALL patients (6 at diagnosis and 5 receiving TKI therapy) and the K-562 cell line were chosen for comparison and sensitivity curves. The tested RT methods were: the RT in-house BIOMED-1 protocol, the RT kit IS CE (Ipsogen) and the RT-Plus kit (Elitech Nanogen). The qualitative-PCRs were performed following: the in-house BIOMED-1 protocol, the Seeplex-Leukemia BCR/ABL (Seegene) and the Alert PHILADELPHIA Oligomix kit (Elitech Nanogen).

Results: As expected, the in-house BIOMED-1 protocol showed excellent reliability, with high correspondence between expected/obtained results, validated by the ABL control gene amplification. Sensitivity with nested PCR was 10^{-5}. This amplification method requires more than 5 hours, due to performing 2 consecutive amplifications and multiple mixes for each PCR. The Seegene kit provided highly reproducible results in a shorter time (3.5 hours), thanks to a single amplification conducted in multiplex-PCR, but did not use ABL as internal control gene and the kit was not CE-IVD marked. The sensitivity was 10^{-4} without production of aspecific bands. The Nanogen kit raised the sensitivity to 10^{-5}, but required the same analytical time of BIOMED-1 protocol and we observed a large number of aspecific transcripts.

Both commercial kits presented some problems with supplied ladders, but Seegene ladder provided a complete panel of positive signals. Both kits provided a strategy for preventing contamination after PCR tubes opening. In all PCR methods we combined the different RT techniques without relevant changes.

Conclusions: The use of BIOMED-1 protocol guarantees the correct characterization of bcr/abl transcript, but this method requires experience to avoid contaminations and a dedicated staff for a single, lengthy analysis. Between the two commercial kits examined, the Seegene kit offers a rapid, reliable and specific test to perform a useful diagnostic screening applicable also in smaller hematologic units/labs.

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Reference

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P045

MINIMAL RESIDUAL DISEASE DETECTION BY NEXT GENERATION FLOW-MRD IN MULTIPLE MYELOMA PATIENTS

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Introduction: Detection of minimal residual disease (MRD) is a sensitive and rapid approach to evaluate treatment efficacy. Negativity of MRD predicts progression-free (PFS) and overall survival (OS) independent of categorical response assessment and patients biology in Multiple Myeloma (MM). In the past 4-6 colours flow cytometry was demonstrated to be an independent predictor of PFS and OS in prospective studies, but it was less sensitive than molecular assays to detect MRD. Recently, two 8 colours tubes panel showed to be able to detect MRD with an increased sensitivity (10-5)(Next Generation Flow-MRD, NGF-MRD). NGF-MRD, applicable to virtually every patients in contrast to molecular approaches that required the development of a patient specific assays, became the preferred method to detect MRD. In MM patients, complete remission (CR) is prerequisite for long term PFS and OS, but little is known about long term remission patients (>5 years) that achieved at least a very good partial remission (VGPR). The aim of our study is to identify the total plasma cell compartment (PCs) in bone marrow (BM) plus the percentages of both normal and neoplastic plasma cells, in long term remission patients using NGF-MRD approach.

Methods: In this study we analyzed 21 bone marrow (BM) samples of MM patients, 11 male, 10 female with the median age 58 (range 55-74 years), 13/21 were predefined as stringent CR (sCR), 4/21 as VGPR and 4/21 were tested while on treatment as group control. Samples were processed within 12h of collection, with a fix and perm protocol and staining using 2- tubes optimized 8-colours antibody panel (OneFlowTM PCST e PCD BD Biosciences).Acquisition was considered adequate when a significant PC population was detected or a minimum of 2 x 106 cells was acquired. Discrimination between phenotypically aberrant (aPCs) and normal (nPC) were based upon the recommendations of the European Myeloma Network. PC populations were identified by gating with CD38 and CD138 positivity after exclude debris, doublets and lymphocytes. nPCs and aPCs are defined based upon a normal or abnormal pattern of expression of others antigens.

Results: In 2/21 cases the samples were not evaluable for the high peripheral blood contamination. Among 19 evaluable patients, only 6/19 showed an MRD+ status. In particular 2/12 in sCR 1/3 in VGPR, 3/ 4 as group control. Interestingly, only 1 /6 patients in long term CR (>5 years) was MRD +. 9/12 patients in CR received an autologous bone marrow transplantation. When evaluating the patients MRD+, the ratio PCn/PCs were 62,5% in sCR patients and 11% in the VGPR patients.

Conclusions: Preliminary results showed that NGF-MRD is applicable in 100% of patients studied but it requires good quality of samples. MRD negative status in the vast majority of long term CR patients could be a first step in defining Myeloma cure. Larger studies are needed to determine the MRD+ status in MM patients with clinical and economic important implications.

P046

THROMBOELASTOGRAM IN CARRIERS OF THROMBOPHILIC MUTATIONS OF PROTHROMBIN (FII) AND FACTOR V LEIDEN

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Factor II (G20210A) and factor V Leiden (G1691A) mutations are more common in young people and in patients (pts) with idiopathic Deep Vein Thrombosis (DVT). In Pulmonary thromboembolism (PTE) mutations have different frequency, in particular Factor V is uncommon (factor V Leiden paradox). The cause of this phenomenon is not clear. It has been hypothesised that the clot stability is increased in pts with factor V mutation, but this has not been unequivocally confirmed by *in vitro* studies. Thromboelastogram (TEG) allows to assess hemostatic and fibrinolytic function. In our study we investigated if TEG could identify any differences between carriers of the two mutations. TEG was performed in 8 carriers of FII (G20210A) mutation, in 11 carriers of FV (G1691A) mutation and in 5 healthy controls, all of them with no history of thrombosis and no therapy with either anticoagulants or antiplatelet agents. TEG was performed with Haemoscope TEG analyzer, based on kaolin procedure. TEG results were within normal range, except for some cases with an increased R value, but average of each parameter is different between two groups. Although the number of pts does not allow statistical analysis, we observed that carriers of G20210A mutation had an increased R value (11.3 vs 9.7), an higher K value (3.43 vs 2.6), a smaller angle (48.5 vs 55.9), a lower maximum amplitude (64.02 vs 68.91), an increased percentage of lysis at 30 and 60 min (1.1 vs 0.3 and 4.7 vs 2.8) and a lower CL (4.87 vs -2.82). These preliminary data suggest that TEG is different in carriers of the two mutations (V Leiden and II mutation) the clot being more stable in carriers of V Leiden with a reduced tendency to embolism. We need a prospective study in a larger cohort of patients and with more advanced methods to confirm these data.

P047

TRANSLOCATION T(8;16) ACUTE MYELOYD LEUKEMIA (AML): CLINICAL, CYTOMORPHOLOGIC, CYTOGENETIC AND MOLECULAR FEATURES OF FOUR NEW CASES

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Introduction: The t(8;16)(p11.2;p13.3) is a rare translocation fusing KAT6A (previously known as MOZ) to CREBBP. It has been associated with de novo and therapy-related AML and is characterized by monocytic or myelomonocytic morphology, extra-medullary involvement, disseminated intravascular coagulation (DIC), erytrophagocytosis and poor outcome. As far as we know, 116 t(8;16)(p11.2;p13.3)-positive AML have been published up to now. Here we report on clinical, morphologic, and molecular-cytogenetic findings in 4 new t(8;16)(p11.2;p13.3) positive AML.

Methods: The 4 patients were retrieved from the Hematology and Bone Marrow Transplantation Units of Perugia and Parma, from 1999 to 2016. They were all females with a median age of 47 years. According to WHO classification diagnosis was AML-M5a in 3 cases (2 de novo and 1 therapy-related -t-AML-) and AML-M4 in the fourth. In this patient, who developed AML while on treatment with dasatinib and etoposide for a p190-positive CML,

with monocytosis, diagnosed 14 months before, we performed serial analysis at four time point during disease monitoring. Molecular cytogenetic analysis was performed on bone marrow samples. Karyotyping was done after GTG or QFQ banding; FISH by applying DNA clones RP11-589C21/5'KAT6A, RP11-461A8/3'CREBBP, and RP11-141K5/RP1-32B1 to study the involvement of MYB in the most frequent accompanying genomic 6q23 gain; RT-PCR to study p210 and p190 BCR-ABL1 transcripts.

Results: The t(8;16)(p11.2;p13.3) translocation was isolated in 3 cases and included in a complex karyotype in the fourth. The KAT6A-CREBBP fusion was demonstrated by FISH in all cases. MYB gain was not detected. In patient no.4, longitudinal studies showed the t(9;22)(q34,q11) (84% of metaphases) and p190 positivity at CML diagnosis; a normal karyotype and p190 positivity at +3 and +9 months; a complex karyotype with p190 negativity at +14 months. All cases had erytrophagocytosis while DIC was never present. Three died of their disease within 7 months; the unique long survivor (+204 months) underwent an identical BMT.

Conclusions: Our study confirmed that the KAT6A-CREBBP fusion identifies a distinct subgroup of de novo or t-AML with monocytoid differentiation and erythrophagocytosis. As it predicts a very dismal outcome the precise molecular diagnosis is mandatory to address the most appropriate treatment, including bone marrow transplantation. Notably, patient no.4 developed a KAT6A-CREBBP positive AML-M4 while on treatment with dasatinib and low dose of etoposide and in molecular remission of the p190+ CML. This intriguing case arises the issue of AML development in Ph-negative cells under TKI treatment. Moreover a t-AML could have been induced by the etoposide. In the last hypothesis a short latency time for t-AML origin is emphasized.

Table 1.

1	F/15	AML-M5a	none	no	13.000	46,XX,i(8;16)(p11.2;p13.3)	204, alive
2	F/47	t-AML-M5a	uterin leyomiosarcoma	Epirubicin, Ifosfamide (12 months earlier)	36.990	46,XX,t(8;16)(p11.2;p13.3)	5, died
3	F/18	AML-M5a	none	no	118.300	46,XX, i(8;16)(p11.2;p13.3)	4, died
4	F/79	AML-M4	CML p190+	low dose etoposide and dasatinib (ongoing)	17.820	46,XX,i(8;16)(p11.2;p13.3)[3]/46,idem,i(2;12)(q10;q24),-17,-17,-22,+mar1,+mar2,+mar3[7]/46,idem,add(1)(p36)[7]	3, died

P048

GENOMIC PROFILE IN FIVE CASES OF T PROLYMPHOCYTIC LEUKEMIA (T-PLL)

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Introduction: T-PLL is a rare aggressive disease characterized by lymphadenopathy, splenomegaly, skin lesions, and poor response to conventional therapy. They shared common abnormalities, i.e. a rearrangement between TCL1, or its homolog MTCP1, and the TCRAD locus; ATM deletion (del); and i(8q). Abnormalities of genes involved in epigenetic transcriptional regulation (EZH2), DNA repair/checkpoint (CHECK2) and the JAK-STAT pathway have also been found. To get insights into T-PLL, we performed an in-depth molecular-cytogenetic characterization of 5 new cases.

Methods: Patients were retrieved in the Hematology and Bone Marrow Transplantation Units of Parma and Perugia. Two cases presented with the typical T-PLL morphologic variant; 2 with the small-cells variant, and 1 with the cerebriform variant. Immunophenotype was consistent with a post-thymic differentiation arrest; in 3 patients high level of CD52 was detected. FISH assays investigated: TCL1 (RP11-952P19), TCRAD (RP11-447G18/RP11-242H9), EZH2 (RP11-420N24), GRIK2/CASP8AP2 (RP11-258B/RP11-81C7), ETV6 (RP11-297N18, RP11-418C2, RP11-434C1), ATM (Vysis), TP53 (RP11-199F11), MYC (Vysis); PLAG1 (RP11-246A9, RP11-1130K23), NCOA2 (RP11-746L20), and CEBPD (RP11-137L5). Double colour FISH experiments assessed clonal configurations in cases with concurrent aberrations.

Results. Three patients had a complex karyotype, with a range of 4-9 aberrations, but only 1 showed a 14q11 involvement, i.e. t(14;14)(q11;q32). This case had double minutes (dms) in all analysed metaphases. All 5 cases invariably bore the TCRAD-TCL1 rearrangement and an ATM del, which, in 4/5, were associated with additional genomic imbalances: TP53 del (=4), 8q gain (=3), EZH2 del (=2), ETV6 del (=1), GRIK2/CASP8AP2 del (=1). TP53 and EZH2 deletions were found as clonal or subclonal abnormalities, while the 8q gain was always subclonal. TP53 del and 8q gain occurred together in 3 cases. Interestingly, in 2 of them, they belonged to independent subclones arising from the stem clone bearing both TCRAD-TCL1 and ATM del.

Conclusion: Besides TCRAD-TCL1 and ATM del, additional recurrent genomic imbalances were linked to T-PLL pathogenesis and/or evolution confirming that ATM disruption and TCL1 activation underlie genomic instability and may occur in independent clones. A clear clonal branching characterized the distribution of TP53, EZH2 and 8q gain in different cases. Our study emphasize EZH2 del in the typical variant of T-PLL. As expected, disease was extremely aggressive in 4 cases (follow-up: 2-13 months). As the long survivor (+70 months), had the small cell T-PLL variant and did not show abnormalities in addition to TCL1 and ATM. The number and the type of T-PLL associated lesions likely impact upon prognosis. Thus, a precise genetic T-PLL characterization would be helpful to fine tune prognostic stratification of patients and to address treatment and monitoring.

P049

CHK1/2 INHIBITION RESTORES THE ANTIPROLIFERATIVE EFFECT OF THE IMMUNOCONJUGATE INOTUZUMAB OZOGAMICIN (CMC-544) ON CD22-POSITIVE CELLS EXPRESSING MUTANT P53

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Introduction: The anti-CD22 investigative drug Inotuzumab Ozogamicin (CMC-544) is a calicheamicin immunoconjugate that has recently received FDA breakthrough designation for ALL. We used immortalized and primary cells derived from CD22-positive malignancies to investigate the molecular pathways involved in the cytotoxic effect of CMC-544.

Methods: We employed BL-2, Sup-B15, Namalwa (CD22-positive) or HL-60 (CD33-positive as control) cell lines. To establish IC50 values, we exposed the above-mentioned cells to logarithmic dilutions of CMC-544 or CMA-676 (anti-CD33). After exposure to CMC-544, alone or in combination with the ChK1/2 inhibitor UCN-01, we analyzed multiple DNA-damage-induced proteins, cell cycle distribution and apoptosis. Cells bearing wild-type or mutant p53 were lentivirally transduced with mutant or wild-type p53 respectively. Treatments with CMC-544 were also repeated on primary cells derived from patients with CD22-positive malignancies.

Results: CMC-544 showed low IC50 values in BL-2 (0.82nM) and Sup-B15 (0.098nM) but not in HL-60 (36.78nM), which were responsive to CMA-676 (0.089nM). However, when we treated Namalwa cells with CMC-544 we found a much higher IC50 value (23.70nM). Exposure of Sup-B15 and BL-2 to CMC-544 caused a G2/M arrest after 24hrs and 60-90% rates of apoptosis after 48hrs. Namalwa cells displayed a 70% rate of apoptosis. However, this result was achieved employing IC50 values 30-240 fold higher than those used for BL-2 and Sup-B15. Namalwa cells also showed a

prolonged G2/M cell cycle arrest. Immunoblots carried out after CMC-544 treatment showed increased ChK1/2 phosphorylation in all cell lines. However, p21 induction was detected in BL-2 and Sup-B15 but not in Namalwa. This result was explained by the presence of a mutant p53 suggesting a p53-independent G2/M cell cycle arrest mechanism. Repeating the same experiments with the sequential combination of CMC-544 and UCN-01, we detected increased apoptotic rates in cells expressing either wild-type or mutant p53 with suppression of G2/M cell cycle arrest after only 12hrs of treatment. Expression of mutant p53 in BL-2 or Sup-B15 increased CMC-544 IC50 values, while a reduction of this value was observed in Namalwa cells after transduction with wild-type p53. We next exposed primary cells derived from 6 patients with DLBCL (displaying wt p53) and 3 patients with leukemized lymphoma (LL, presenting mutant p53) to CMC-544. We found CMC-544 IC50 average values of 65.91nM for DLBCL and 1176.60nM for LL. Hence, our findings suggest that p53 status plays a pivotal role in the cytotoxic effect of CMC-544.

Conclusion: CMC-544 displays potent anti-proliferative activity against both immortalized and primary CD22-positive cells. p53 mutational status might represent a predictive biomarker for CMC-544 efficacy both *in vitro* and *in vivo*. Associating a ChK1/2 inhibitor with CMC-544 may restore the compound's activity in CD22-positive malignancies displaying p53 mutations.

P050

ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES BINDING TO NEUTROPHIL ELASTASE (HNE ANCA) OCCURRING AFTER COCAINE EXPOSURE MAY INDUCE AN ACQUIRED FORM OF CYCLIC NEUTROPENIA

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Introduction: Cocaine exposure may lead to the occurrence of clinical manifestations associated to ANCA and lupus anticoagulant (LAC) positivity. Cocaine adulterant levamisole is known to be responsible for distinct disorders ranging from cutaneous necrotizing vasculitis to life-threatening agranulocytosis (AGC). When associated to vasculitis, AGC tends to recover with drug withdrawal, however little is known about the long-term clinical course of this condition. The exact pathogenesis of levamisole-induced disease is currently uncertain, but an immune-mediated process is favored rather than a direct toxicity. Cyclic neutropenia is a congenital disorder due to the mutation of the gene coding for neutrophil elastase (NE), leading to a "gain-of-function" in the protein causing cellular toxicity in neutrophil precursors.

Methods: We report 3 cases of cocaine-associated cyclic ANCA-positive AGC, treated with immunosuppressive therapy (IST) because of recurrent disease in the absence of further cocaine exposure. In order to explore the pathogenetic mechanism of this disorder, we performed indirect immunofluorescence and capture and nickel ELISAs to detect HNE ANCA.

Results: 3 pts (1 M/2 F, aged 36, 41 and 45, respectively) w/o a family history of neutropenia, developed recurrent episodes of AGC associated to acute tonsillitis, oral mucositis, or perianal ulcers, after cocaine use. Known causes of secondary AGC were not detected. ANCA testing was positive in all pts: MPO in 1 pt, PR3 in 1 pt, MPO and PR3 in 1 pt. LAC was available and positive in 2 pts. The BM examination showed a maturational arrest of myeloid precursors. A relapse of the AGC after an initial self-recovery was observed in all cases, independently from further cocaine use, with a spontaneous cyclical drop of neutrophil counts, refractory to GCFS. Thus IST was started. In 1 case HD prednisone was administered for 2 mo, with success. After 2 mo the patient relapsed, and was put on azathioprine, obtaining a new prolonged remission. The other 2 pts achieved remission after 3 and 2 courses of HD IvIg, respectively (Figure 1). Pts' median fu was 27 months. We hypothesized that an immune mechanism ought to be responsible for the premature apoptosis of neutrophil precursors leading to cyclic hematopoiesis. To support this hypothesis we tested pts' sera for HNE ANCA, directed against NE. We observed a significant titer of HNE ANCA in all 3 pts before starting IST. In 1 pt the fu titer was available and decreased during remission.

Conclusions: Our study provides the first evidence of a potential correlation between acquired cyclic neutropenia and HNE ANCA, possibly induced by exposure to cocaine, which would trigger an immune-mediated process leading to a chronic disease. It further demonstrates the efficacy of IST to induce remission in cyclic cocaine-associated ANCA-positive neutropenia, showing how the drug withdrawal is not sufficient to prevent disease relapse and life-threatening infections.

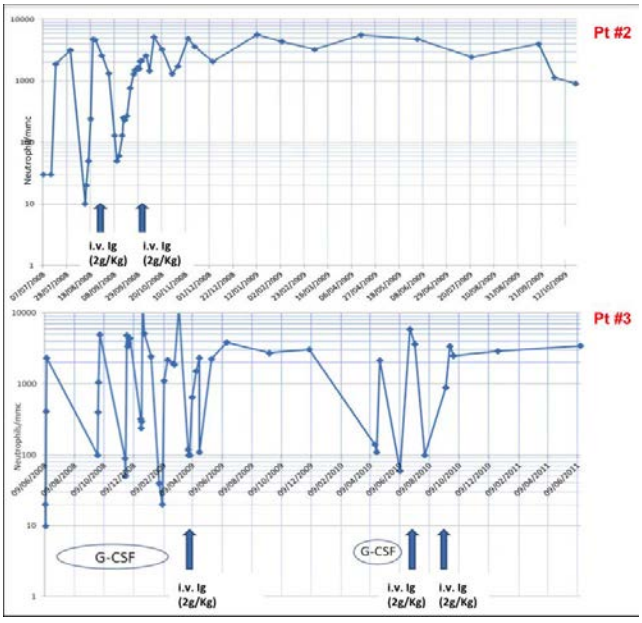

Figure 1.

P051

DIGITAL PCR FOR MINIMAL RESIDUAL DISEAS ANALYSES IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

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Introduction. Minimal residual disease (MRD) is the most important prognostic factor in adult and childhood Acute Lymphoblastic Leukemia (ALL), driving clinical decision at fixed time points in clinical protocols. Huge efforts have been done to standardize reliable techniques for MRD detection. These techniques are based on identification of Ig/TCR clonal rearrangements at diagnosis followed by Real Time quantitative PCR (RQ-PCR) using clone specific oligonucleotides for subsequent MRD analyses. MRD level is expressed as leukemia reduction based on a dilution curve of diagnostic material. Therefore, the availability of high amount of DNA at disease onset is mandatory to ensure MRD evaluation. New technologies with absolute leukemia level evaluation would be of interest for MRD measurement.

Methods: Droplet digital PCR (ddPCR) is an innovative technology in which PCR reaction is performed on single DNA mole-

cules in separate reaction units (droplets). Patient-specific assays developed for MRD evaluation with RQ-PCR were used for MRD detection and quantification with this novel approach. Follow-up samples were derived from 5 ALL patients selected based on material availability and RQ PCR assay high sensitivity (10-5). Results were compared to those obtained by the gold standard RQ PCR method standardized within the collaborative European MRD study group.

Results: Twenty-one follow-up samples (10 bone marrow and 11 peripheral blood) were studied with ddPCR using one (9 samples) or two (12 samples) RQ-PCR assay for a total of 37 analyses. We obtained concordant results in 31 out of 33 evaluations (94% concordance) with 2 follow-up samples resulting positive in RQ-PCR and negative in ddPCR. In these two cases the positivity level was low and outside the quantitative range (10-4) of RQ-PCR assays. A very good concordance in MRD levels was found in the 18 samples positive for the two techniques: samples positive within the 10-1/10-4 range with RQ-PCR had similar MRD level with ddPCR; samples positive outside the quantitative range in RQ PCR had a 10-5 or less MRD level in ddPCR. Diagnostic material and mononuclear cells (MNC) were also added in each assay to evaluate the specificity of the ddPCR. All but 1 MNC sample gave concordant results with one positive droplet present in ddPCR in a MNC sample negative by RQ-PCR.

Conclusions: Our results demonstrate a good concordance between MRD levels obtained with ddPCR and RQ-PCR. Therefore, ddPCR could be a valid alternative to study MRD also in patients without a high quantity of diagnostic material. However, RQ-PCR appears to be still superior in revealing low MRD load. The low number of sample tested in parallel with the two techniques could account for this latter result and requires additional analyses on a large number of coupled samples. International efforts are in progress to define experimental condition and interpretation rules for this novel attractive technique.

P052

REGULATORY T CELLS (TREGS) GMP MANUFACTURE FOR CLINICAL USE: PHENOTYPIC AND FUNCTIONAL ANALYSIS OF CRYOPRESERVED/THAWED NORMAL TREGS

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Treg based immunotherapy may be beneficial in several immune mediated diseases including Graft Versus Host Disease (GVHD). The possibility of cryopreserving TREGs might lead to the administration of multiple doses, thus potentially increasing their efficacy in chronic diseases. However, there are few and controversial data on functionality of TREGs after cryopreservation of freshly isolated cells. Here, we evaluated the phenotype (classical TREGs markers and specific markers for suppressive cells) and the inhibitory capacity of thawed TREGs by using three methods of thawing. TREGs were purified under GMP condition from leukapheresis of normal donors (N=2) by double depletion (CD8 and CD19) followed by CD25 enrichment using the immunomagnetic CliniMACS System (Miltenyi Biotec). The cells were cryopreserved in saline solution containing 10% Human Serum Albumin (HSA) and 10% DMSO with a controlled-rate freezing. Three distinct thawing strategies were tested: A) directly thawing at 37°C; B) thawing at 37°C followed by the drop-by-drop addition of an equal volume of a cold thawing solution (saline, 12% ACD-A, 10% HSA); C) thawing at 37°C followed by the drop-by-drop addition of an equal volume of a pre-warm thawhing solution. Cell viability was assessed by trypan blue and 7-AAD staining. Phenotype was evaluated on fresh and thawed TREGs. Thawed autologous T effector cells (Teff) were used in MLR assays. The two apheresis (#1 and #2), contained $166.95x10^6$ and $193.71x10^6$ CD4+CD25+CD127low cells, respectively. The absolute number of freshly isolated CD4+CD25+ CD127low cells was $94.61x10^6$ (#1) and $102.73x10^6$ (#2) with a post enrichment recovery of 57% and 53% respectively. The percentage of CD4+CD25+FoxP3+ cells was 71% for #1 and 55% for #2. The freshly isolated TREGs from both apheresis efficiently reduced by about 50% the anti-CD3/CD28-induced proliferation of autologous Teff, at a 1:2 TREGs-Teff ratio. Mean viability of TREGs thawed with the three different methods above mentioned was 76±13%, 72±8% and 91±1% (method A, B and C, respectively), as assessed by trypan blue, and 75±8%, 74±5% and 87±1% (method A, B and C, respectively), as evaluated by 7-AAD staining. As expected, irrespective of thawing methods, the viable cells were almost totally CD4+CD25+ (97±1%). The percentage of CD4+CD25+FoxP3+ cells was 54±7% (#1) and 55±3% (#2). Thawed TREGs were all CD45RA- and CD62L+ (#1: 39±5%; #2: 32±5%), CD15s+ (#1: 2.7±0.3%; #2: 2.9±0.2%), HLA-DR+ (#1: 5.8±2 %; #2: 13.4±0.6%) and CD31+ (#1: 8.8±2.4%; #2: 0.7±0.1%). Notably, viable thawed TREGs ware able to induce inhibition (around 50%) of autologous Teff cells in a TREGs:Teff ratio 1:2 as freshly isolated TREGs. We demonstrate that thawed freshly isolated normal TREGs maintain a stable phenotype and a good suppressive efficacy. The method C was the most effective in preserving cells viability. However, the viable cells obtained with the three thawing methods showed comparable phenotype and function.

P053

WT1 GENE: A NOVEL SPLICING VARIATION INVOLVING THE EXON 5 IN ACUTE MYELOID LEUKEMIA PATIENTS

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Introduction. The Wilms' tumor 1 gene (WT1) is a transcription factor that plays an important role in cellular development and survival [1,2]. It has both tumor suppressor and oncogenic role in several hematopoietic malignancies, including acute myeloid leukemia (AML) [3,4]. WT1 gene is formed by 10 exons; a total of 24 different isoforms resulting from alternative splicing events, alternative start-codons and RNA editing were reported. About the splicing events, the most significant variations are: inclusion or skipping of exon 5 (exon5+/exon5-) and alternative splice donor sites between exons 9 and 10 (KTS+/KTS-) [1-4]. The aim of our study was to recognize both known and novel WT1 SNPs with impact on splicing process.

Patient and methods. We studied 13 AML patients recruited from two different Italian geographic areas: Marche (Clinic of Hematology, Hospital-University Company "Ospedali Riuniti", Ancona-group1) and Basilicata (Hematology Division, San Carlo Hospital, Potenza-group2). Patient gDNA was isolated at AML onset. WT1 consensus splicing regions were genotyped by means of PCR amplification with specific primers (for the coverage of exon-intron boundaries) and direct sequencing. Bioinformatics software (BlastN and Mutation Surveyor) were used for DNA variant analysis compared to the gene RefSeq (NG_009272.1). In silico analysis was also performed with the aim to predict new potential splicing sites (Human Splicing Finder 3.0).

Results. We reported a novel WT1 variation, such as a cytosine insertion between 23992 and 23993 sites (g.23992_23993insC) residing within the natural donor site of exon 5 (nt 24002…24052). The splice site was predicted to be broken in presence of our variation. The insertion was found in 5/8 patients (62,5%) of group 1 and

interestingly absent in the second group of patients.

Conclusions. The novel variation could improve the background for a molecularly-based risk ALM assessment and the treatment stratification, also based on patient geographical area. Other WT1 isoforms could increase the panel of known variants and, consequently, the complexity of gene biology. Our preliminary finding is to be validated including in our study a larger group of AML patients, belonging to other Italian regions.

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P054

DNMT3A MUTATIONAL STATUS IN 219 AML PATIENTS: ATYPICAL MUTATIONS RESPOND BETTER THAN TYPICAL ONES

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Introduction: Acute myeloid leukemia (AML) is a heterogeneous disorder which prognosis depends on the presence or absence of cytogenetic aberrations and several molecular alteration profiles. In AML with normal karyotype, several molecular markers with prognostic impact can be identified, such as FLT3, NPM1, MLL, WT-1, DNMT3A, CEPBα, c-KIT, TP53, NRAS, KRAS, IDH1, IDH2, TET2, SETD2 and EZH2. In particular, DNMT3A mutations are associated with poor prognosis and so we decided to identify its mutational status in our AML patients treated since 2013.

Methods: We analyzed 219 AML patients at diagnosis for DNMT3A with Sanger Sequencing. 34/219 (15,53%) patients revealed a DNMT3A mutation: 21 R882H (61,77%), 6 R882C (17,65%), 3 R882S (8,82%), 1 W860R (2,94%), 1 P779R (2,94%) and 2 patients carried non-point mutation, never reported to our knowledge. One resulted to have a deletion of exons 21 and 22 and a W795*, while the other one presented a 36bp insertion in exon 22. These two alterations were not found in the germ-line samples, analyzed by SNPs arrays and NGS approaches. The karyotypes of our patients were heterogeneous: 13/34 were normal karyotype, 7/34 had a complex karyotype and 3 patients carried one or two alterations. 11/34 patients had a not available karyotype. We also evaluated the mutational status of FLT3, NPM, IDH1, IDH2 and TP53 mutations. 4/34 (11,76%) were ITD+, 13/34 (38,24%) were NPM1+, while 8/34 (23,53%) were both ITD/NPM1+. Moreover, we found that 5 patients (14,71%) carried an IDH1 mutation, 9 (26,47%) were IDH2 mutated, while 20/34 (58,82%) were wild-type. TP53 was evaluated in 22/34 patients and only 2 patients with CK were mutated.

Results: We monitored the bone marrow of the two patients with atypical DNMT3A mutations at different time points by NGS runs performed with the Roche Junior 454, while the other 32 patients were monitored by Sanger Sequencing. We found that the patients who carried DNMT3A point mutations never become wild-type despite the remission of the other molecular markers, while both the patients with the atypical mutation reduce significantly the allelic burden of the mutation. The first one reduce the W795* from 31,70% at diagnosis to 1,77% after 5 months, while the deletion could not be detected anymore. The other patient, instead, presented the insertion at 27,50% at diagnosis and then decreased to 15,05% after two months of therapy and become negative after 4 months.

Conclusion: Both these two patients show, after 6 months from diagnosis, a clinical RC, in contrast with the other patients with typical point mutations, where the molecular remission was reached later or never. Based on these results we can believe that these two atypical mutations could be considered as MMR markers, contrary to the point mutations, but further samples have to be evaluated in the follow-up.

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P055

SUCROSOMIAL IRON IS EFFECTIVE IN TREATMENT OF YOUNG WOMEN WITH CHRONIC INFLAMMATION AND SIDEROPENIC ANEMIA AND REDUCES INFLAMMATORY MARKERS IN VIVO AND *IN VITRO*

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Introduction: Liposome has a described anti-inflammatory effect and transports its content directly in blood, beyond enteric wall. 9 patients (4 with systemic erythematous lupus, 3 with mixed connectivitis, 2 with rheumatic fibromyalgia), median age 32 years (R27-42), median Hb 8.5 g/dl (R8-10), saturation of iron binding capacity <20%, with a median ferritin level of 100 ng/ml (R90-250), ESR 35 mm/1st hour (R22-95), CRP 18 mg/I (R12-24), normal B12 and folate, received sucrosomial iron 60 mg/day orally for 3 months. After treatment, patients showed a median hemoglobin level of 11.5 g/dl (R10.5-12), a median ferritin level of 260 ng/ml (R 190-280), a ESR decrease to a median value of 8 mm/1st hour (R 3-10) and a median CRP 3 mg/I (R2-4). Then sucrosomial iron is safe, effective, well tolerated, effective in increase hemoglobin level and reduce inflammatory markers in correction of sideropenic anemia of chronic inflammatory disease of young women. Aim of this study is to understand if sucrosomial iron inhibits expression of IL-6 in U937 cell line (monocyte cell line) and CRP and hepcidin in HepG2 cell line (hepatoma cell line).

Methods: U937 and HepG2 cells at a cell density of 650,000 cells/ml were suspended in incomplete FBS without antibiotics and glutamine containing 20 ng/ml of LPS from E. Coli. The compounds used for the experiments were: sucrosomal Iron (Sideral ®), liposomal matrix, iron pyrophosphate. The Iron content of the compounds was 30 mg/mL. After 6, 18 and 24 hours, supernatants from the wells were collected, centrifuged 10 min at 1000g, transferred to a new tube and stored at -20°C to perform hepcidin, IL-6 and CRP analysis. Marker analysis was performed using commercial available ELISA kits at 6, 18 and 24 hours. Before to treat cells with the selected compounds, they were tested to assess their dissolution proprieties.

Results: The levels of IL-6 produced by cells were higher at 18 and 24 hours after the treatment. At 6 hours the differences between the treatments were barely visible. At 18 and 24 hours the treatment with only LPS showed high level of IL-6 content. The Sideral showed an effect of reduction of IL-6 at 18 and 24 hours, while the pyrophosphate iron had an increasing effect on IL-6 at these time points. The treatments with matrix had a more evident

effect of reduction of IL-6. The levels of hepcidin were increased at every time points when the cells were treated with only LPS. With remaining treatment there were no specific evidence of hepcidin levels increase. At 24 hour there were high levels of CRP with LPS and pyrophosphate iron and low levels with sucrosomal Iron and liposomal matrix

Conclusion: Sucrosomal Iron and liposomal matrix showed to be able to reduce inflammation markers *in vitro* and *in vivo* in correction of sideropenic anemia of chronic inflammatory disease of young women.

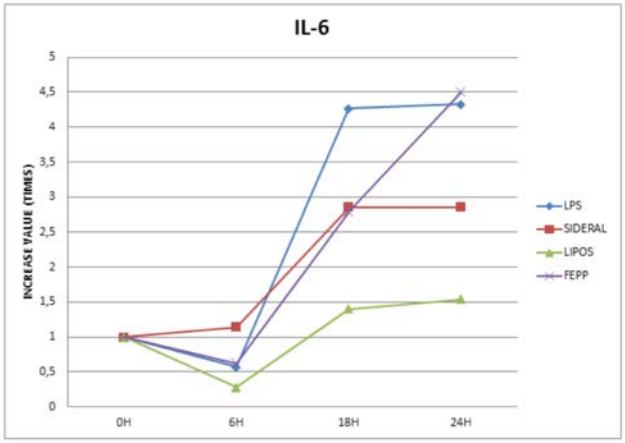

Figure 1.

P056

ASSESSMENT OF THE INTERLABORATORY VARIABILITY AND ROBUSTNESS OF JAK2 V617F MUTATION ASSAYS: A STUDY INVOLVING A CONSORTIUM OF 19 ITALIAN LABORATORIES

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In chronic myeloproliferative neoplasms, the JAK2V617F allele burden (AB) is crucial for diagnosis and prognosis assessment, and also for disease monitoring after allogeneic stem-cell transplantation. A plethora of techniques for JAK2V617F determination is used over different laboratories, which differ both in specificity and sensitivity. Given the need to provide reliable and comparable molecular results, the standardization of molecular techniques is fundamental. The aims of this study were to evaluate the inter- and intra-laboratory variability in JAK2V617F quantification in 19 laboratories, to identify the most robust assay for the standardization of the molecular test and to allow consistent interpretation of analysis results. The study was developed in 3 different rounds (Figure 1) and involved 19 Italian molecular laboratories. Both reagents and DNA samples were provided by Werfen-IL SpA and QIAGEN. Statistical analysis was carried out by QIAGEN/Bologna University. In the 1st round, we aimed to investigate the inter-laboratory variability on different mutation loads.

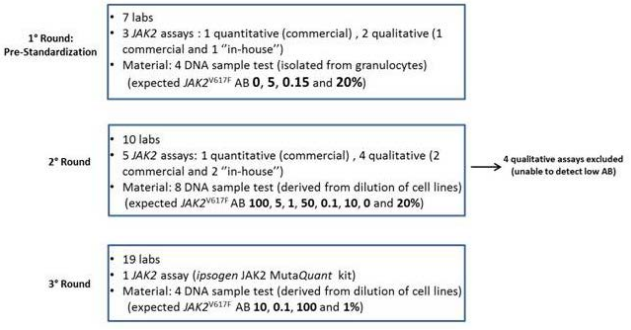

Figure 1.

All laboratories using a quantitative approach were able to determine the expected JAK2V617F AB. Conversely, laboratories using a qualitative approach did not detect the positivity of samples with a low AB. To further investigate the inter-laboratory variability on low-positive samples, we developed a 2nd round, in which 3 additional laboratories were included. Each laboratory performed 2 runs with ipsogen JAK2 MutaQuant kit and 2 runs with their "in-house" method. None of the laboratories using qualitative methods were able to detect low AB samples, while quantitative results by ipsogen JAK2 MutaQuant kit showed only a small variability among laboratories at low AB (0.1 and 1%; CV =0.42 and 0.24, respectively). The 3rd round was intended to confirm the robustness of the ipsogen JAK2 MutaQuant kit. The study was therefore extended to 9 additional laboratories. "Homemade" methods were excluded and all laboratories performed 2 runs with the ipsogen JAK2 MutaQuant kit. Quantitative results were well reproducible across all mutation loads. Only one labo-

ratory failed to quantify 0.1% sample in one run. Importantly, all laboratories clearly distinguished between the 0.1 and 1% mutated samples (0.1 and 1%; CV =0. 64 and 0.77, respectively). The study firstly demonstrated that a qualitative approach is not sensitive enough to detect the JAK2V617F mutation at a low (‚â§1%) burden. Conversely, the ipsogen JAK2 MutaQuant kit resulted highly efficient and sensitive in the quantitative detection of all mutation loads. This study sets the basis for the creation of an Italian network of molecular laboratories focused on the diagnosis of MPNs, including not only JAK2V617F, but also CALR and MPL mutations. The network will aim to identify/standardize the most efficient and cost-effective techniques for the evaluation of these mutations, so to produce reliable and reproducible molecular data.

Acute Leukemia 2

P057

AN HPLC AND 1H NMR STUDY OF THE CYTARABINE DEGRADATION IN CLINICAL CONDITIONS TO AVOID DRUG WASTE, DECREASE THERAPY COSTS AND IMPROVE PATIENT COMPLIANCE

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Cytarabine, the 4-Amino-1-(β-D-arabinofuranosyl)-2(1H)-pyrimidinone, (ARA-C), is an antimetabolite cytidine analogue used worldwide as key drug in the management of leukaemia. As specified in the manufacturers' instructions, once the components – sterile water and ARA-C powder – are unpackaged and mixed, the solution begins to degrade after 6 hours at room temperature and 12 hours at 4 °C. To evaluate how to avoid wasting the drug in short-term low dose treatment regimens, the reconstituted samples, stored in the dark at 25° and 4° C, were analyzed every day of the test week by reversed phase high-performance liquid chromatography (RP-UHPLC) and high-field nuclear magnetic resonance spectroscopy (1H NMR). All the samples remained unchanged for the entire week, which corresponds to the time required to administer the entire commercial drug package during low-dose therapeutic regimens. The drug solution was stored in a glass container at 4 °C in an ordinary freezer and drawn with sterile plastic syringes; during this period, no bacterial or fungal contamination was observed. After one month, the samples presented evidence of a degradation product (0.8% of starting material), identified as 1-(β-D-arabinofuranosyl)-pyrimidine-2,4-(1H,3H)-dione (ARA-U).

Our findings provide evidence of an optimal physico-chemical stability and microbiological sterility of ARA-C solution stored for one week in the dark, at 4°C. This encourages the use of the reconstituted drug for the time required for short-term multi-dose treatments, avoiding drug waste, patient stress and hospital crowding. Moreover, it seems possible to leave in the same container surplus of different ARA-C packages, improving the cost-effectiveness of the treatment without affecting its efficacy and safety. An additional advantage is the fact that patients are able to have the treatment administered at home.

Our results show that a solution of reconstituted ARA-C could be employed for a longer period that what suggested by the manufacturers. In fact, patients could receive a safe aliquot to be used at home for short-term treatments, thus optimizing the use of aliquot residues and avoiding vial manipulation and the production of special waste material.

P058

POST INDUCTION WT1 VALUES AND WT1 ONSET/POST INDUCTION RATIO ARE USEFULL TO STRATIFY ACUTE MYELOID LEUKEMIA PATIENTS

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Introduction: WT1 is an important marker for minimal residual disease detection in acute myeloid leukaemia (AML), but its role in risk stratification is not clear. We speculate a risk stratification method based on WT1 post induction values (WT1-PI) and WT1 onset/post induction ratio (WT1-R).

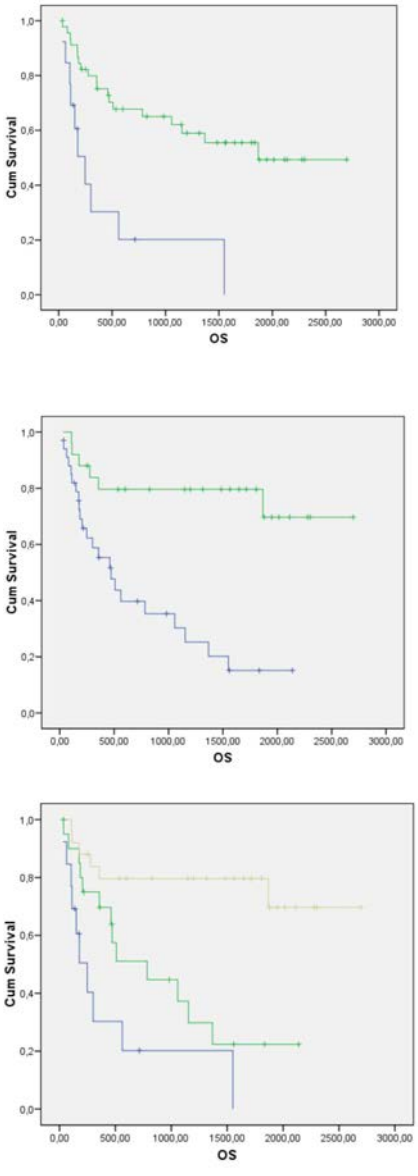

Figure A. OS in patients with WT1 PI >80 (blu line) and WT1 PI <80 (green line). Figure B. OS in patients with WT1-R < 38,55 (blu line) and WT1-R> 38,55 (green line). Figure C. Cumulative OS in patients with: - WT1-PI > 80 and WT1-R<38,55 (blu line, unfavourable); - WT1-PI <80 and WT1-R <38,55 (green line, intermediate); - WT1-PI<80 and WT1-R >38,55 (yellow line, favourable).

Methods: Between 2008 and 2015, 25 bone marrow samples (BM) of healthy subjects (14 male, 11 female, median age 58, range 23-89) and 65 BM of AML receiving induction therapy (30 male, 35 female, median age 64, range 21-89) were screened for WT1 using RQ-PCR. At onset WT1 was measured in all patients, while WT1-PI values were measured in 60 cases (it was not possible in 3 patients because of early death and in 2 cases because of technical reasons). Of the 60 evaluable patients after induction therapy, 47 were in complete remission (CR), 7 were in partial remission (PR), 6 had progressive disease (PD).

Results: WT1 median value at onset in AML patients was 969.7 (range 0.3-34497.0); it was 8.30 (range 0.78-78.8) in healthy subjects. ROC analysis indicated 80 as optimal "threshold" of WT1 value (area of 0.9439, sensitivity of 0.89 and specificity of 1) to discriminate normal from AML bone marrow. We stratified the 60 patients, evaluable after induction, according to this value. 15/60 had a WT1-PI > 80: 8 were in CR, 3 in PR and 4 with PD. 11 of these 15 patients (73%) died 5 months after onset (range 1- 52 months). 45/60 had WT1-PI value <80 (39 were in CR, 4 in PR, 2 with PD) and 42% (19/45) died within 12 months (range 1-62). We calculated WT1-R in all 60 patients. According to ROC analysis, 38.55 is the cut off level indicative of probability of survival (sensitivity 0.60 and specificity 0.83). We then performed survival analysis based on WT1-PI alone, WT1-R alone and both. Using both the criteria, it is possible to share patients in three groups: unfavourable (patients with WT1-PI >80 and WT1-R <38.55), intermediate (patients with WT1-PI <80 and WT1-R <38.55) and favourable (patients with WT1-PI <80 and WT1-R >38.55).

Conclusion: Together WT1-PI and WT1-R seem to be an optimal tool to stratify AML patients in favourable, intermediate or unfavourable groups for survival. These, together with other evaluations could guide physicians in planning therapy.

P059

T(8;12)(Q13; P13) B-COMMON ACUTE LYMPHOID LEUKEMIA IN A PATIENT WITH PREVIOUS T(8;12)(Q13; P13) MYELOPROLIFERATIVE NEOPLASM

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Introduction: Qualitative and quantitative abnormalities of chromosomes 8 and 12 have been described in different hematological malignancies such as acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), acute lymphoid leukemias (ALL) and biphenotypic leukemias. The t(8;12)(q13; p13) results in fusion of the transcriptional repressor ETV6 (TEL) gene and the transcriptional coactivator NCOA2 (TIF2). Germline mutations of the ETV6 gene have been described in MPN charaterized by thrombocytopenia and megaloblastic anemia prone to development of ALL. Here we report of one patient diagnosed with MPN characterized by t(8;12)(q13;p13) that evolved into ALL bearing the same chromosome rearrangement.

Methods: A 62 year old male was admitted in the emergency department for dyspnea in February 2014. Physical examination was negative and lab exams showed thrombocytopenia (80.000/mmc), macrocitic anemia (Hb 9,5 g/dL, MCV 104 fL) and mild leukocytosis (WBC 11.530/mmc of which N 8160/mmc, L 1640/mmc, M 470/mmc, E 660/mmc,>B 160/mmc and blasts 410/mmc). The bone marrow biopsy supported such MPN/MDS. The karyotipe revealed a traslocation between chromosomes 8 and 12(46, XY, t(8;12)(q13;p13)[23]/46, XY [1]). The final diagnosis was MPN/MDS (IPSS 2), BCR-ABL negative, JAK2-v617 wild type. Because of the blurred clinical picture, the patient was discharged with a "watch and wait" program. In August 2015 he was admitted for general worsening of clinical state, fatigue and peripheral edema. Blood tests revealed thrombocytopenia (80.000/mmc), macro-

cytic anemia (Hb 10,5 g/dL, MCV 104 fL) and leukocytosis (189.000/mmc). Bone marrow biopsy with immunophenotypic analysis concluded for B common ALL without expression of T-cell or myeloid markers. The BCR-ABL was negative. The cariotype described the same structural rearrangement present in the previous MPN (46, XY, t(8;12)(q13;p13)[20]) without additional abnormalities. The patient was enrolled in one national clinical trial for Ph negative ALL and begun the chemotherapy program. Unfortunately he died during induction for pulmonary invasive aspergillosis.

Results: view case description

Conclusions: To our knowledge this is the first report of one case of chronic MPN/MDS with t(8;12) evolved into clonally related t(8;12) ALL. We can speculate that this translocation, involving the ETV6 (TEL) gene, is related to the leukemogenesis of adult ALL. A stringent follow up schedule in patients with t(8;12)(q13;p13) MPN/MDS with thrombocytopenia and macrocitic anemia is strongly advisable because of the risk of ALL transformation.

P060

GONADOTROPIN RELEASING HORMONE AGONIST TO PREVENT CHEMOTHERAPY-INDUCED OVARIAN DAMAGE IN ACUTE LEUKEMIA PATIENTS

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Introduction: Anticancer treatments have improved survival rates among patients with hematologic malignancies and long-term side effects of chemotherapy (CHT), like infertility have become a major issue. As recommended by ASCO and ESMO, embryo/oocyte cryopreservation are standard strategies for fertility preservation in women, while other strategies like pharmacologic protection of gonads and gonadal tissue cryopreservation are considered experimental techniques. There is little data regarding the effects of leukemia CHT on ovarian reserve, specifically in adult women.

Methods: In order to evaluate the impact of acute leukemia and its treatment on fertility and the efficacy of temporary ovarian suppression with gonadotropin-releasing hormone agonist (GnRHa) or Oral contraceptive pill (OCP) during CHT, we retrospectively analyzed a population of 35 premenopausal women of median age 37.8 years (range 20-47), treated for AML (65.7%; 23 patients) or ALL (34.3%; 12 patients), since 2005 to 2015. The efficacy of temporary ovarian suppression was evaluated by the return of spontaneous menstruation and rate of pregnancy after the end of CHT.

Results: 33 of 35 patients received GnRHa (leuprorelin acetate monthly administration) alone or in association with OCP during CHT, in order to prevent ovarian damage. Conversely, 1 patient died before starting administration and the other one had previously undergone hysterectomy. Among these 33 patients, only 19 could be evaluated in term of preserved ovarian function defined as regular menses whereas 11 (33%) early died and 3 (9%) were lost in follow up. 9 patients (47%) underwent HSCT, namely 2 autologous and 7 allogeneic. The mean duration of GnRHa therapy was 7.6 months (range 1-19). 10 patients (53%) resumed menses within a median of 3-4 months after termination of treatment. Pregnancy occurred in 2 patients (10%).

Discussion: Current options for fertility preservation in cancer patients during CHT may not be feasible in acute leukemia because of the impossibility to delay CHT and the potential risk of recurrence of disease after reimplantation of contaminated ovarian tissue. The use of GnRHa in young female patients to suppress pituitary gonadotropin secretion and cyclic ovarian function is still considered experimental and of debated potential efficacy. In our study, despite the limited patients cohort and the absence of hormone levels monitoring, the use of GnRHa alone or with OCP is safe, effective in preventing heavy vaginal bleeding and not associated with delay of CHT. The return of spontaneous menstruation seems to be more associated with treatment intensity than with age. Indeed, none of the patients who underwent HSCT resumed regular menses, as opposed to patients treated with CHT alone. This is a critical issue because HSCT frequently represents the best treatment for adults with leukemia. Further studies are necessary to assess the role of GnRHa in fertility preservation.

P061

CD34 EXPRESSION CUT-OFF VALUE FOR PREDICTION OF PML-RARα ISOFORMS IN ADULT ACUTE PROMYELOCYTIC LEUKEMIA

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Introduction: Acute promyelocytic leukemia (APL) is characterized by t(15;17)(q22;q12), resulting in production of the fusion transcript PML-RARα: the short bcr3 and the long bcr1 are the most frequent isoforms. We analyzed 38 diagnosed APL patients, with a mean age of 46 ys (range 17-80), 25 males and 13 females, to identify immunophenotypic markers for prediction of PML-RARα isoforms.

Methods: Immunophenotypic analysis was performed on bone marrow cells by APL panel using FACSCantoII cytometer and BDFACSDiva software. A positive reaction was defined when ≥20% of APL cells expressed more fluorescence intensity than control cells. As other investigators, we have used a 10% cut-off for CD34 positivity. All cases were confirmed to have t(15;17) by karyotyping analysis and PML-RARα isoforms (bcr1/bcr3) by RT-PCR assay. Student's t-test or Mann-Whitney test was performed for comparisons of means and Fisher's exact test was used to compare categories. Cut-off value for the marker was selected using receiver operating curves (ROC). Multivariate analysis was performed using Logistic Regression. P values lower than 0.05 were considered statistically significant (STATA 12.0).

Results: The short bcr3 and the long bcr1 isoforms of PML-RARα were identified in 14 pts (36.8%) and 24 pts (63.2%), respectively. Patients with bcr3 form were significantly younger (mean age, 39 vs 52 ys, P=0.012) and exhibited a greater median percentage of CD34 (5.6% vs 0.8%, P<0.001) compared to pts expressing bcr1 form. Association between bcr3 expression and CD34 positivity (21%) was assessed by Chi-square analysis (P=0.007). A similar relationship was found between CD2 positivity (26%) and bcr3 form (P=0.033), despite no significant difference was observed in median percentages of CD2 expression in the two cohorts. Both CD34 and CD2 positivity were associated with the M3v subtype (P=0.049 and P<0.001, respectively), with a higher median leukocyte count (17.2 vs 1.4 x 10^9/L, P=0.018 and 22.1 vs 1.6 x 10^9/L, P=0.001, respectively) and with a greater percentage of peripheral blood leukemia promyelocytes (83% vs 33 P=0.033 and 81% vs 25% P=0.002, respectively). Moreover, CD34 and CD2 positivities were associated each other (P=0.001). By calculating ROC, the best cut-off of CD34 expression percentages to discriminate PML-RARα isoforms was 3.1% (AUC=0.85, 95% CI: 0.73 to 0.98). This cut-off value provided a sensitivity of 71% and a specificity of 87%, while the reported cut-off value for CD34 positivity (≥10%) demonstrated a lower sensitivity (43%) and a higher specificity (96%). Multivariate analysis in which CD34 positivity (cut-off ≥3.1%) was combined with CD2 positivity showed CD34 positivity as an independent predictor for discriminating PML-RARα isoforms (P=0.047).

Conclusions: CD34, as a sole marker, is able to discriminate the short bcr3 and the long bcr1 isoforms of PML-RARα. We suggest a cut-off value of 3.1% for CD34 in order to maximize sensitivity and specificity.

P062

FRONT-LINE CENTRAL VASCULAR ACCESS DEVICES IN ACUTE LEUKEMIAS - PERIPHERALLY INSERTED CENTRAL CATHETER (PICC) VERSUS TRADITIONAL CENTRAL VENOUS CATHETER (CVC): A PHASE IV RANDOMIZED TRIAL (NCT02405728)

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Our Hematology Department is conducting a Phase IV randomized trial. We compare PICCs versus traditional CVCs as front-line venous access device in patients with acute leukemias undergoing chemotherapy for remission induction (ClinicalTrials.gov, NCT02405728; ongoing). Primary endpoint is the occurrence of catheter-related bloodstream infections and/or catheter-related thrombosis. Secondary endpoints are the occurrence of other complications, such as pneumothorax or catheter occlusion, and patients'quality of life. From April 2015, 152 patients with acute leukemia planned for remission induction chemotherapy were randomly assigned (1:1) to PICC (Arm A) or traditional CVC (Arm B) (Table 1). Inclusion Criteria were: age >18 years, expected survival > 4 weeks, and need of central venous access device (long-term : ≥ 4 weeks). All insertions were followed by ultrasonography assessments and chest X-ray. 152 patients (median age 47 years, r.13-82), 130 acute myeloid leukemia (AML) and 22 acute lymphoblastic leukemia (ALL), were randomized in two arms. In the Arm A, 76 PICCs were inserted in 76 patients (median age 51.5 years, r.19-82; 17 females, 59 males) suffering from AML (70) or ALL (6). Double lumen PICCs (5 Fr) were inserted in 70 patients, single lumen PICCs (4 Fr) were inserted in 5 patients, and triple lumen PICC (6 Fr) was inserted in 1 patient. 68 PICCs were inserted in the right basilica vein, 5 PICCs were inserted in the left basilica vein and 3 PICCs were inserted in the left brachial vein. In Arm B, 76 traditional CVCs were inserted in other 76 patients (44 males, 32 females) suffering from AML (60) or ALL (16). 45 CVCs were inserted in subclavian vein and 31 CVCs were inserted in jugular vein.

Table 1.

	Arm A : PICCs	Arm B : CVCs
N° patients	76	76
Median age	51.5 (19-82)	42.5 (13-74)
M/F	59/17	44/32
Acute myeloid Leukemia	70	60
Acute lymphoblastic Leukemia	6	16
Place of insertion	68 right basilica veins 5 left basilica veins 3 left brachial veins	45 subclavian veins 31 jugular veins

Overall, the median duration of in situ catheter placement was 5 months : 6 months (r.3-12) in the arm A vs 3 months (r.1-10) in the arm B. In the arm A, catheter-related thrombosis occurred in 8 patients (6 basilica veins, 2 brachial veins) and catheter-related bloodstream infections in 4 patients (4 coagulase-negative staphylococci; of them, 2 meticillin-resistants). In the arm B, 20 cases of catheter-related thrombosis (7 subclavian veins, 13 jugular veins) and 15 cases of catheter-related bloodstream infections (10 enterobacteriaceae; 5 coagulase-negative staphylococci, and, of them, 3 meticillin-resistants) were observed. Thus, PICCs were significantly associated with fewer major complications compared with traditional CVCs (catheter-related thrombosis: 10.5% in the arm A vs. 26% in the arm B, p=0.01 by $\chi 2$ test; catheter-related bloodstream infections: 5% in the arm A vs. 19% in the arm B, p= 0.007 by $\chi 2$ test). The preliminary observations of this ongoing Phase IV randomized study, focusing on front-line use of central venous access device in an high-risk hematological population, suggest that the use of PICC represents an advance in terms of decrease of complication rate and improve of quality of life for patients with acute leukemia.

P063

SURVIVAL ANALYSIS OF 409 CONSECUTIVE PATIENTS WITH NEWLY DIAGNOSED ACUTE MYELOID LEUKEMIA TREATED WITH INTENSIVE INDUCTION THERAPY, WITH OR WITHOUT THE ADDITION OF GEMTUZOMAB-OZAGOMICIN (GO)

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Background: Young patients affected by non APL-Acute Myeloid Leukemia (AML) achieve complete remission (CR) using conventional induction chemotherapy in about 55-70%. The addition of Gemtuzumab Ozogamicin (GO) demonstrated to improve clinical outcome, in terms of CR rates. We retrospectively evaluated and compared the efficacy of different induction schedules, in terms of CR rates and Overall Survival (OS), administered to two groups of AML patients. Group 1 (n=139) was treated with a GO (MyFLAI or MyAIE schedules); group 2 (n=270) received a non-GO based regimen including or not Fludarabine (FLAI, FLAN, FLAG, 3+7 or DAE).

Methods: From 1997 to 2014, 409 patients with newly diagnosed AML were treated in 3 Italian Institutions. According to karyotype (performed in 392/409 patients), FLT3 (available for 244/409 patients), and NPM1 mutational status (available for 157/409 patients), based on the NCCN-2013 risk stratification criteria, 35.2% of the patients were considered at High Risk (HR) (31.6% and 36.4% in the two groups, respectively) and 7.6% at low risk (LR) (7.8% and 7.0%, respectively).

Results. The complete remission (CR) rate after induction was 81.4% and 70.4% for Group 1 and 2, respectively (p=0.008). Deaths during induction (DDI), occurring in the first 50 days from 1st line therapy, were 4/139 (2.9%) in Group 1 and 22/270 (8.1%) in Group 2. Patients treated with GO showed a better OS than patients of Group 2; the 5-years OS in the two groups was 54.01% and 34.9%, respectively, and different according to age (54.0% and 34.9% respectively (p<0.001) in patients <60 years, 30.2% and 13.5% respectively (p=0.001) in patients >=60 years). Notably, the analysis on subgroup of HR patients showed a significantly better OS in Group 1 than in Group 2 (p=0.007, 5-year OS 47.7%; 21.0% respectively) and EFS.

Conclusions: We may therefore conclude that adding GO to any induction regimen is an independent and strong predictor of better OS and higher CR rate. This new approach to AML front line treatment could be recommended in SR and HR AML patients, related to karyotype abnormalities, in which showed an advantage in terms of OS if compared with other standard regimens.

Acknowledgments: Work supported by ELN, AIL, AIRC, Progetto Regione-Università 2010-12 (L.Bolondi), FP7 NGS-PTL project.

P064

SPECIFIC CHROMOSOMIC ALTERATIONS CONFER THERAPY RESISTANCE IN A COHORT OF 49 PATIENTS WITH NEWLY DIAGNOSED ACUTE MYELOID LEUKEMIA TREATED WITH INTENSIVE CHEMOTHERAPY

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Introduction. Intensive induction chemotherapy in non-M3 young Acute Myeloid Leukemia (AML) patients is represented by the association of an antracycline and Cytarabine. Some treatment regimens including fludarabine or the addition of Gemtuzumab Ozogamicin (GO) as a third or fourth drug, proved to give a benefit in terms of CR rates.

Aims of the study. In a group of 49 patients treated with intensive chemotherapy, we evaluated chromosomal abnormalities with SNP 6.0 and Cytoscan HD (Affymetrix) in order to improve conventional cytogenetic analysis and discover novel chromosomic aberrations related to clinical data and therapy response.

Patients and Methods. From 2001 to 2014, 489 patients were treated in our Institution. Among those, in 49 newly diagnosed AML patients (median age 54 (range 19-71)), SNP microarray based-genotyping were performed and then analyzed by Nexus Copy Number™ v7.5 (BioDiscovery) and R Development Core Team According to karyotype, FLT3 and NPM1 mutational status, 55.9% of the patients were considered at High Risk (HR) and 4.1% at low risk (LR). Ten patients had secondary AML. Patients were treated with induction schemes including MyFLAI, MyAIE, FLAI, FLAN, FLAG, 3+7 or DAE.

Results. The CR rate after induction was 87.8% (43/49 patients). Deaths during induction (DDI), occurring in the first 50 days from 1st line therapy, were 1/49. The median OS was 135 months, the 5-years OS in our patients was 55,1%. Patients treated with GO showed a non-statistical trend toward a better OS than patients treated with other regimens (median OS not reached vs 133 months, respectively). We explored the alterations found by SNP array in our patients searching for novel markers of therapy resistance. We found a median of 192,5 total copy number aberrations (range 72-1071): a median of 145,5 total copy number aberrations in responding patients group (RPG), and a median of 361 total copy number aberrations (p=ns) in non-responding patients group (NRPG). We compared the frequency of detected aberrations in RPG and in NRPG with Fisher's exact test. We found that PIK3CA, Gain chr3:178,927,088-178,929,550 (p=0,0016), SMAD4, Gain chr18:48,573,154-48,573,255 (p=0,0166) and several other gene's loci (CASC18, TCF12, UTY, GRB10, ZFY) are significant aberrations in NRPG compared with RPG.

Conclusions. We identified a number of genes with significant aberrations in NRPG, particularly PIK3CA, a protein-coding gene involved in cell proliferation and metabolic pathway with interaction with HRAS/KRAS and EGF, and SMAD4, a transcription factor activated by TGF-beta. Those 2 genes were found overexpressed in other solid tumors. We suppose that those genes may be involved in a hyper-proliferative pathway that underlies a mechanism of chemo-resistance.

Acknowledgments Work supported by ELN, AIL, AIRC, Progetto Regione-Universit^ 2010-12 (L.Bolondi), FP7 NGS-PTL project.

P065

AZACITIDINE IN MAINTENANCE THERAPY AFTER COMPLETE REMISSION POST INDUCTION CHEMOTHERAPY IN OLDER PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Background: We retrospectively analyzed the safety and the outcome of older Acute Myeloid Leukemia(AML) 28 patients (de novo or secondary)observed from 2013 to 2015 in 5 hematological institutions from the Apulia Region unfit for allogeneic steam cell transplant.

Aim: Observing the risk of cumulative toxicity treatment, therapy related death and infections related mortality prolonging intensive chemotherapy during consolidations in older patients , we decided to treat a setting of patients with age up to 65 , which reached complete remission with a cycle of induction (anthracycline-cytarabine regimens), with a maintenance therapy based of azacitidine 75 mg/m^2/day for 7 days every 28 days until progression of disease.

Patients: The study included 28 AML patients (14 male, 14 female), median age 74 years (range 65-82), 18 patients de novo (64%) and 10 secondary(34%), PS<2 ECOG .The molecular and cytogenetic evaluation was performed in 79% of patients and in 10% and 21% was adverse molecular and cytogenetic analysis, respectively.

Results: The overall response (complete remission 64%+partial remission 32%) during treatment of induction and maintenance was 96% and median overall survival was 12 months with a range from 2 and 90 months. About safety of subcutaneous azacitidine maintenance 3 patients (10%) experienced grade 3/4 myelosuppression and 9 patients (32%) developed infections and 1 died during septic shock.

Discussion: No statistical difference was demonstrated between achievement complete remission and overall survival and cytogenetic stratification , respectively. While we observed an higher number of partial remission or no response patients in secondary AML group (p<0.001).

Conclusion: After 0btaining complete remission following standard induction chemotherapy in older patients, the hypometilant during maintenance might prolong the remission and improve the survival, preserving the quality of life of this setting of AML patients.

P066

EARLY PERIPHERAL BLAST CLEARANCE CORRELATES WITH OVERALL SURVIVAL AND TIME TO RECURRENCE IN PATIENTS WITH ACUTE MYELOID LEUKEMIA

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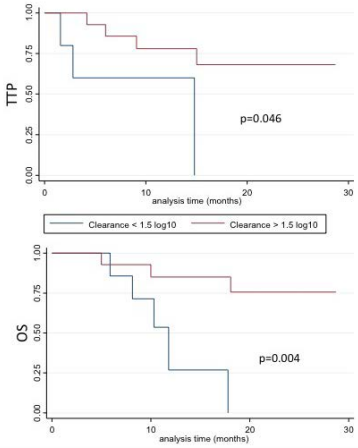
Introduction: Biological pretreatment factors affect the prognosis of patients (pts) with Acute myeloid leukemia (AML). In addition, response to induction chemotherapy, assessed by residual bone marrow (BM) leukemic blasts during aplasia, has significant prognostic impact (Kern, 2003). Recently, the prognostic impact of early clearance of peripheral blood blasts (PBB) seemed to predict the efficacy of induction therapy (Gianfaldoni, 2008- Vainstein, 2014).

Methods: We analyzed 21 pts (M:F 10:11; median age 63 y [range 26-73]) who received a new diagnosis of AML at our Institution. Eighteen pts underwent induction therapy with idarubicine, cytarabine, and etoposide (ICE), while 3 pts received cytarabine plus daunorubicine (3+7). Six-color multiparameter flow cytometry (MFC) detected a leukemia associated immune-phenotype (LAIP) for blasts BM at diagnosis in 19 out 21 pts. PBB clearance, evaluated by MFC was arbitrarily considered as achieved when a 1.5-log10 reduction in the 1st day-to-4th day ratio of leukemic blast cells was documented. Minimal residual disease (MRD) persistence at post-consolidation was defined as LAIP-positive cells higher than 0.035% after acquiring at least 250000 events. Contingency tables were analyzed by the Fisher's test. Time to relapse (TTR) and overall survival (OS) were analyzed by Kaplan-Meier method and compared by the log-rank test. Univariate survival analysis was performed by the Cox proportional hazard regression.



Patients characteristics

	positive clearance	negative clearance
n° pts	14	7
medium age (years)	56	64
ELN risk:		
favorable	6/14 (43%)	3/7(42%)
intermediate (I-II)	5/14 (36%)	2/7 (29%)
adverse	3/14 (21%)	2/7 (29%)
CT ICE	13/18 (72%)	5/18 (28%)
CT 3+7	1/3 (33%)	2/3 (67%)
CR post induction CT	100,00%	71,40%
MRD negative	7/9 (78,00%)	2/9 (22,00%)
Relapse	4/14 (29%)	5/7 (71%)
OS (months)	**not reached**	**11.8 (5.88-n.r.) ***
TTR (months)	**not reached**	**6.13 ***
Follow up (median in months)	21	10.3

* p value < 0.05

Fig1 TTP and OS depending on PBB clearance (> or < 1.5 log10; line red or blue) and the table with Patients characteristics. In the graph of the OS were included also the two pts who had not responded to induction therapy (not included in the graph of TTP)

Results: Nineteen out 21 pts achieved complete remission (90,5%). Early PBB clearance was obtained in 14/21 pts (67%) [PBB clearance range: 0.35-3.36]. The induction regimen (ICE vs. 3+7) did not influence the clearance of blasts. The main characteristics of pts subdivided according to PBB clearance is summarized in Fig 1. There was no difference in ELN risk stratification. PBB clearance was associated with significantly better OS [median survival not reached vs 11,8 months; P 0.004] (Figure 1). Furthermore, we demonstrated a longer OS in those pts achieving a deeper clearance. In particular, in univariate analysis every 1-log increase in PBB clearance predicts a 69% decrease in the mortality risk (HR: 0.31, p=0.016, CI: 0.12-0.80). Moreover PBB clearance predicted a longer TTR [median not reached vs 6,1 months; P 0.046] (Figure 1) but the overall frequency did not differ between PBB clearance positive and negative pts (4/14 vs 5/7; P 0.159). While PBB clearance was not associated to MRD achievement [P 0.35] (Table in Fig 1), MRD negativity was, also, significantly correlated with OS (P 0.011).

Conclusions: With the limitations of the small number of patients studied, PBB clearance seems an early and effective post-treatment prognostic factor for both OS and TTR in pts with AML, independently from the well known predictive power of postconsolidation MRD achievement. Further studies will be necessary to define whether early PBB clearance may affect the therapeutic decisions in AML pts.

P067

AZACITIDINE IN THE REAL LIFE TREATMENT OF OLDER UNFIT AML PATIENTS: A REPORT OF TWO ITALIAN CENTERS

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Introduction: Azacitidine (Aza) efficacy and safety in older patients with de novo or secondary AML not considered eligible for intensive chemotherapy (IC) and/or hematopoietic stem cell transplantation (HSCT) were evaluated.

Methods: Eligible patients were age > 65 years with intermediate or poor-risk cytogenetics AML unsuitable for IC or subsequent HSCT (ECOG PS score > 2 ; age > 75 years, WHO grade 3 toxicities after induction therapy). Aza 75 mg/sqm per day was administered sc for 7 consecutive days per 28-day treatment. After 4 and 8 treatment cycles BM aspirates and PB smears were evaluated. Global OS from the start of Aza to death of any cause was evaluated and OS was evaluated according to patients subgroups (age > 75 years, gender, BM blasts < 20% vs > 20% at baseline, prior MDS). Hematologic response were defined by IWG criteria. Stable disease was defined as not meeting criteria for any other response. Transfusion independence for patients who were transfusion-dependent at baseline was also assessed.

Results: Between January 2011 and December 2015 a total of 87 AML patients received Aza treatment in two Institutions (UD 40 cases, SGR 47 cases). More than half of all patients (72.4%) were age > 75 years and/or intermediate or poor-risk (54 %) cytogenetic. Median BM blasts at baseline were 27%. Patients received a median of 4 cycles (range 1-20) of Aza. Thirty-nine patients (45%) received 6 or more treatment cycle and ten (11.5%) received 12 or more cycles. The median duration of follow-up was 11 months. Overall response rate (CR+ Cri+ PR) was 36.7% and 30 patients (42.2%) had stable disease during treatment. Median OS was 10 months (range 9.0-12). By study end, 65 deaths (75%) occurred. Responder patients showed a better OS than non responders (20 vs 10 months, P<0.001). OS in patients with stable disease was 10 months. In multivariate analysis (age >75 years, male vs female gender, BM blasts < 20% vs > 20% at baseline, prior MDS) only a prior MDS was correlated with a worse OS (8.5 vs 11 months in de novo AML, p<0.02). Independence or reduced need for transfusions

were obtained in 6 (14.2%) and 12 (28.5%) patients respectively and a stable need for transfusions was observed in 12 cases (28.5%). Only 8 (9.2 %) patients (according to the advanced age of the whole population) received a subsequent alloHSCT.

Conclusions: In these older unfit AML patients, real life Aza treatment confirms a benefit in terms of both CR and OS, regardless of the BM blast counts. In this experience Aza treatment was generally well tolerated and adverse events were consistent with the safety profile of the drug.

P068

THE FOXC1 TRANSCRIPTION FACTOR IS DOWN-REGULATED IN ACUTE PROMYELOCYTIC LEUKEMIAS AND MODULATED BY TREATMENT WITH ATRA

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Introduction: Acute promyelocytic leukaemia (APL) is characterized by the aberrant oncogenic PML-RARα protein which blocks myeloid differentiation at the promyelocyte stage. FOX genes encode transcription factors able to regulate many developmental processes during embryogenesis and tissue differentiation, and are involved in several fundamental processes of cell differentiation in adults. FOX genes are de-regulated in several tumors and their increased expression has been shown to correlate with poor differentiation and dismal prognosis. Recently, the FOX family member FOXC1 has been reported to be overexpressed in 20% of human AML.

Methods: We studied FOXC1 mRNA expression in bone marrow mononuclear cells (BM-MNC) isolated from 54 patients with newly diagnosed AML (27 APL and 27 other AML subtypes). Samples from 11 APL patients collected at the time of remission following consolidation therapy (AIDA n=9; ATRA-ATO n=2) were also studied. DNA methylation of exon 1 of the FOXC1 gene was investigated by pyrosequencing. The NB4, NB4-R4, HL60, U937 and HEK cell lines were used for *in vitro* treatment and methylation studies. FOXC1 expression was also studied during *in vitro* differentiation of CD34+ progenitors obtained from human cord-blood.

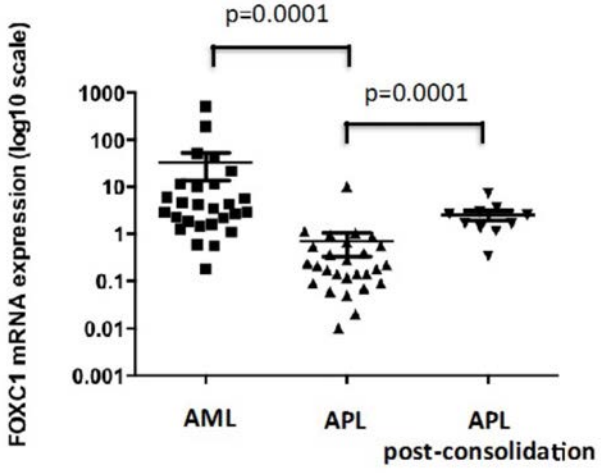

Figure 1. FOXC1 mRNA relative expression in 27 AML, 27 APL and 11 APL post-consolidation.

Results: FOXC1 mRNA levels were significantly lower in primary APL samples, as compared to other AML subtypes (p=0.0001, figure 1). In addition, FOXC1 protein levels were significantly lower in APL, as compared to other AML subsets, (p=0.0008). FOXC1 expression significantly increased in marrow samples collected from APL patients at the time of remission following consolidation treatment (p=0.0001, Figure 1). In keeping with observations in primary samples, compared to other cell lines, NB4 t(15;17)-positive cells exhibited lower FOXC1 expression levels, which increased following treatment with ATRA. Notably, in the NB4-R4 cell line, a RA-resistant derivative of NB4, FOXC1 levels remained stable after ATRA treatment. Conversely, myeloid cell differentiation from cord-blood CD34+ cells was associated with a marked decline of FOXC1 expression. Reduced FOXC1 mRNA and protein expression was consistently associated to DNA hypermethylation (p=0.0093) in APL samples only, while the methylation status observed after consolidation treatment was similar to normal BM pattern. Similarly, FOXC1 gene methylation was higher in NB4 cells than in other cell lines, but did not change upon ATRA treatment. FOXC1 methylation was functional, since treatment of the HL-60 cell line with decitabine induced FOXC1 demethylation and up-regulated FOXC1 mRNA and protein expression.

Conclusions: FOXC1 is down-regulated in primary APL samples and is induced by differentiation with ATRA. Both methylation and ATRA may play a significant role in the regulation of FOXC1 expression.

Myeloproliferative Disorders and Chronic Myeloid Leukemia 2

P069

IMATINIB MESYLATE AND CARDIAC FUNCTION

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Cardiotoxicity of second and particularly third generation TKI is well known, while cardiotoxic effects of Imatinib mesylate has never been properly demonstrated. A group of CML patients in long term treatment with Imatinib, with no pause in their treatment and with no known previous cardiac problems was investigated by cardiac echography. A case control study was performed, with 23 people in treatment with Imatinib vs 23 people selected from a list of people with similar fisiological characteristics who also had a echography.

Both the groups were composed by 18 men and 5 women,whose age were 47.6 ± 14.3 (control) and 47.7 ± 14.2 (Imatinib, for at least 4 years at full dose or with highest doses entered the study). The functional and anatomic cardiac values of these 2 groups were compared and we found some statistical differences, in particular in these echocardiographic parameters: Imatinib patients have an increasing in Inter ventricular septal thickness (10.1 ± 2.0 vs 9.0 ± 1.2, P<0.03), Left ventricular internal end-diastolic diameter (51.5 ± 5.0 vs 48.0 ± 4.7, P<0.02), Left ventricular internal end-sistolic diameter (35.6 ± 5.9 vs 32.7 ± 4.2, P<0.06), left ventricular mass(g) (178.3 ± 66.5 vs 140.5 ± 27.1, P<0.02), left ventricular mass index (41.5 ± 14.1 vs 32.7 ± 6.2,P<0.01) and left atrium (mm) (40.7 ± 6.4 vs 33.8 ± 4.9,P<0.0001). These anatomical differences can be compared to those of people affected by cardiopathies : in fact, this anatomical profile is that of patients with Left Ventricular Hypertrophy. In our series Imatinib-related cardiotoxicity is present, unrelated to the dose and the length of treatment, even if not in a serious grade. An ecocardiographic study should be performed at start of IM treatment and repeated during the follow-up especially when the first examination shows abnormalities. It could be useful to perform in every the LMC-centers a case control study with a long term follow-up to clarify the real impact of IM on heart failure.

Variabile	Control n=23	Glivec (Case) n=23	P
Sex (M/F)	18/5	18/5	NS
Age (years)	47.6 ± 14.3	47.7 ± 14.2	NS
BMI (Kg/m2)	23.5 ± 2.4	26.5 ± 4.4	<0.005
Sistolic BP (mm Hg)	124.1 ± 13.2	131.4 ± 16.0	NS
Diastolic BP (mm Hg)	76.4 ± 6.1	76.0 ± 10.5	NS
Mean BP (mm Hg)	92.3 ± 7.8	94.5 ± 11.7	NS
Heart rate (bpm)	76.3 ± 18.4	78.8 ± 16.8	NS

BMI= Body Mass Index
BP= Blood Pressure

Variabile	Controlli	Glivec	P
IVST (mm)	9.0 ± 1.2	10.1 ± 2.0	<0.03
PWT (cm)	8.4 ± 1.2	8.4 ± 1.5	NS
LVIDD cm)	48.0 ± 4.7	51.5 ± 5.0	<0.02
LVIDS (cm)	32.7 ± 4.2	35.6 ± 5.9	<0.06
LVM (g)	140.5 ± 27.1	178.3 ± 66.5	<0.02
LVMI (g/m2.7)	32.7 ± 6.2	41.5 ± 14.1	<0.01
RWT	0.35 ± 0.07	0.32 ± 0.06	NS
EFS (%)	31.9 ± 5.6	30.9 ± 8.7	NS
LA (mm)	33.8 ± 4.9	40.7 ± 6.4	<0.0001
E (cm/s)	0.74 ± 0.21	0.72 ± 0.13	NS
A (cm/s)	0.65 ± 0.21	0.71 ± 0.19	NS
E/A ratio	1.20 ± 0.40	1.07 ± 0.35	NS
DT (ms)	192.0 ± 34.3	184.2 ± 54.0	NS
IVRT (ms)	78.3 ± 13.6	79.3 ± 19.9	NS

IVST= Inter ventricular septal thickness
PWT= Posterior wall thickness
LVIDD= Left ventricular internal end-diastolic diameter
LVIDS= Left ventricular internal end-sistolic diameter
LVM= left ventricular mass
LVMI= left ventricular mass index
RWT= relative wall thickness
EFS= ejection fractional shortening
LA= left atrium
DT= deceleration time
IVRT= isovolumic relaxation time

P070

PROTEIN KINASE CŒ EXPRESSION IN PRIMARY MYELOFIBROSIS PLATELETS CORRELATES WITH CARDIOVASCULAR EVENTS

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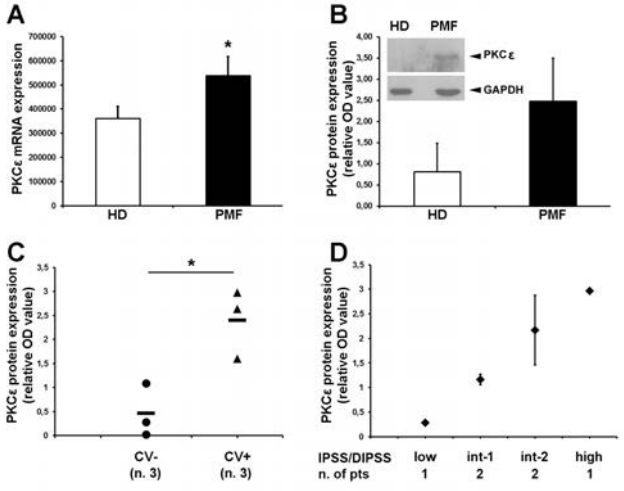
Introduction: Among the three classic Philadelphia-negative MPNs, myelofibrosis (MF)-either primary (PMF) or arising from a previous PV or ET-is characterized by profound alterations in megakaryocytopoiesis and thrombopoiesis, such as hyperplasia of abnormal megakaryocytes (MK), aberrant platelet production and consequent variable platelet count with altered hemostatic function (Barbui T, 2010). Although all three diseases are typified by increased risk of thrombotic events, data from the literature concern primarily PV and ET. Indeed, *ex-vivo* platelets studies showed enhanced reactivity of ET and PV platelets (Viallard JF, 2002; Villmow T, 2002; Bermejo E, 2004; Falanga A, 2005). We previously demonstrated that the protoncogene protein kinase Cε (PKCε) is over-expressed in platelets from patients with acute myocardial infarction and accounts for their increased reactivity (Carubbi C. 2012). Additionally, we recently showed that PKCε overexpression plays a crucial role in PMF MK impaired differentiation and that its levels correlated with disease burden (expressed by IPSS/DIPPS risk category) (Masselli E, 2015, Carubbi C, Haematologica 2016). On these bases, we assessed PKCε expression in platelets from PMF patients, investigating a potential correlation with the thrombotic risk–represented by a history of cardiovascular events-and the aggressiveness of the disease.



Methods: Peripheral blood samples from 6 PMF patients (all JAK2V617F pos) and 3 healthy donors (HD) were collected in Na-citrate tubes. PKCε mRNA and protein levels were determined by qRT-PCR and western blot analysis in platelets purified as described by Carubbi C, 2012. Patients were stratified according to the history of cardiovascular events (CV+ and CV-) and the IPSS/DIPSS risk category. Statistical analysis was performed by t-test.

Results: PMF platelets showed significantly higher mRNA levels of PKCε as compared to HD (536,698.3±79,859.2 vs 360,943.6±50,123.7, respectively; p=0.042 panel A; ΔCt was expressed with reference to the platelet marker CD41). Protein analysis confirmed PKCε over-expression in PMF platelets, almost reaching statistical significance (2.48±1.02 vs 0.81±0.68 of HD, p=0.059, panel B; data are expressed as relative PKCε/GAPDH

OD values).

We then found that platelets from PMF patients at high thrombotic risk for prior cardiovascular events (CV+) display significantly higher levels of PKCε as compared to CV- (2.4±0.71 vs 0.46±0.55, p=0.02, panel C). Finally, similarly to what observed in PMF magakaryocytes, we showed a trend in terms of correlation between PKCε platelets levels and IPSS/DIPSS risk category, with the lowest levels in low-risk patients and higher levels in high-risk patients (panel D).

Conclusion: Our data indicate that PMF platelets display an increased expression of PKCε which is associated to a history of cardiovascular events, suggesting that PKCε over-expression may account for PMF platelet altered reactivity and function.

P071

WT1 ANALYSIS IN CHRONIC MYELOID LEUKEMIA (CML) PATIENTS

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Introduction: Diagnosis and monitoring of CML patients is based on bcr/abl evaluation by RQ-PCR. ELN guidelines show that bcr/abl inferior to 10% after 3 months of therapy and bcr/abl inferior to 1% after 6 months of therapy is an optimal response. Different clinical parameters are used to predict patients' prognosis at onset in the three most commonly used score indexes. No other useful tools have been identified. WT1 gene is over expressed at onset in several diseases including 80% of Acute Myeloid Leukaemia cases (AML): gene's levels decrease during disease remission and are low in healthy people. WT1 is over expressed also in case of AML relapse. No data are available on WT1 expression in CML patients.

Methods: We evaluated 28 patients with CML referred to our centres between 2009 and 2015. RT-PCR and RQ PCR on RNA were performed to quantify bcr/abl and WT1 in the peripheral blood of all patients at onset and after 3, 6, 9 and 12 months of therapy (median follow up 19 months, range: 9-70). The type of transcript was p210 b2a2 in 6 pts, p210 b3a2 in 19 and both in 3 pts. Sokal risk was low in 9 pts, intermediate in 12, high in 7. 14 patients received Imatinib, 3 Dasatinib and 11 Nilotinib as first line therapy.

Results: 9/28 of patients (32%) had high levels of WT1 (WT1-H) at onset. In 7 of these patients (78%), Sokal risk was high/intermediate; in 10/19 WT1-L patients (53%) Sokal risk was high/intermediate. 8/9 WT1-H patients were evaluable for bcr/abl at three months of therapy: 1 had bcr/abl > 10%, 4 between 1 and 10% and 3 lower than 1%. 17/19 WT1-L patients were evaluable at three months: 2 had bcr/abl > 10%, 3 between 1 and 10%, 12 lower than 1%. At sixth months, 80% of WT1-H and 17% of WT1-L patients showed bcr/abl levels>1%. Only 3/9 WT1-H patients achieved and maintain at least a MR3. 4 patients never achieved MR3, 2 achieved and subsequently lost MR3: these 6 patients shifted to second line therapy. 17/19 WT1-L pts achieved and maintain at least MR3; 1 patients lost MR3 end only 1 patient never achieved MR3. These 2 patient shifted to second line therapy. Patients status is different in the two groups. In the WT1-H group, 1 patient died, 4 failed to achieve a response, 1 achieved a MR2, 1 a MR3, 2 a MR4. In the WT1-L group, 1 died, 1 achieved a MR2, 7 a MR3, 10 a MR4.

Conclusion: In our study cohort, WT1-H patients seem to have a worse outcome than WT1-L, in terms of MR3 and shift to second line therapy because of failure: 67% of WT1-H patients vs 10% of WT1-L patients needed to change therapy. WT1 could be a prognostic marker at onset.

We need to study a larger number of patients to confirm this hypothesis.

P072

THE HOCT1/ABCB1 APLOTYPE IS ASSOCIATED WITH COMPLETE CYTOGENETIC RESPONSE AND TOLERABILITY TO IMATINIB IN PATIENTS AFFECTED BY CHRONIC MYELOID LEUKEMIA: A NEW MATHEMATICAL APPROACH

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Introduction: Drug transporters, such as hOCT1 (SLC22A1), ABCB1, and ABCG2 play a control of both intracellular and systemic plasma concentrations of imatinib. Our group showed that the hOCT1 c.480C>G polymorphism conditions higher rates of adverse events and shorter EFS. Analogously, the ABCB1 polymorphisms have been related to a worse prognosis, but this item is still debated.

Methods. The aim of this study was to develop an accurate mathematical model for imatinib that would include pharmacogenetic and clinical data. Such a model, which was obtained in an unsupervised way through a factorial analysis, was used to further investigate the role of time-independent covariates on the drug efficacy and toxicity in the context of the TIKlet protocol (ClinicalTrials.gov code: NCT01860456).

Results. Fifty-three CML patients receiving imatinib, 30 males and 23 females, were enrolled at the Hematology of Pisa and Orbassano (TO), Italy. The following polymorphisms were assessed: rs72552763 [MI420I], rs12208357 [c.181C>T] and rs683369 [c.480C>G] (hOCT1), rs1128503 [c.1236C>T], rs2032582 [c.2677G>T/A] and rs1045642 [c.3435C>T] (ABCB1), rs4149117 [c.334G>T] (SLCO1B3). Patients were grouped according to the absence (wild-type) or the presence (heterozygous or polymorphic homozygous) of at least one polymorphic allele. The Factor Analysis of Mixed Data (FAMD) was used to identify an ordered sequence of the most important dimensions along which data were distributed and to quantify the correlations between each dimension and the set of covariates. We found that both the complete cytogenetic response and the maximum toxicity were in strong relationship with the combination of hOCT1 rs683369 [c.480C>G] and ABCB1 rs1045642 [c.3435C>T] polymorphisms. Indeed, genotypes identical for both loci (i.e., wild/wild or polymorphic/polymorphic) exhibited a longer time to CCyR than those with mixed types (wild/polymorphic or polymorphic/wild) (p=0.013). Moreover, since the drug tolerability has a strong incidence on the discontinuation of the therapy, we studied also whether the time to the onset of toxicities would be related to any considered variable. The hOCT1 c.480C>G and ABCB1 c.3435C>T genotypes were significantly associated with the maximum degree of toxicity (p-value = 0.022). In particular, patients with mixed hOCT1 c.480C>G and ABCB1 c.3435C>T genotypes displayed higher levels of toxicity. The time of manifestation of the cramps toxicity was shorter in females than in males (5.3 vs 22.6 months). On the other hand, time to manifestation of edema toxicity was associated with a combinations between male gender and ABCB1 c.3435C>T.

Conclusions. The novelty of the present study is the adoption of an unsupervised technique to investigate any possible relationship

between pharmacodynamics, pharmacogenomics, and clinics. This approach allowed the identification of several covariates that could be claimed as significant factors in drug efficacy and tolerability.

P073

EFFICACY AND SAFETY OF FIRST-LINE TREATMENT WITH NILOTINIB IN ELDERLY CHRONIC MYELOID LEUKEMIA PATIENTS

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Background. In chronic phase (CP) chronic myeloid leukemia (CML) nilotinib (NIL) has shown better efficacy compared to imatinib, but it has been associated to a higher incidence of arterial thrombotic events (ATEs). Elderly patients treated with NIL may be particularly at risk for ATEs, which ultimately may impact on their outcome.

Aim. To investigate the efficacy and safety, particularly the cardiovascular safety, of NIL in elderly CP CML patients.

Methods. We analyzed 345 patients with CP CML enrolled in clinical trials of the GIMEMA CML WP investigating NIL as first-line treatment. Patients were treated with: NIL 400 mg BID (n=73); rotation of NIL 400 mg BID / imatinib 400 mg OD (3-month periods for each drug)(n=123); NIL 300 mg BID (n=149). The median follow-up was 58 months. The median age was 53 years. We analyzed in detail the response rates, events and outcome of 89 patients ≥ 65 years of age; these patients were also compared to the 245 patients < 65 years. Definitions: Major molecular response (MMR): BCR-ABL≤0.1% (IS), with > 10.000 ABL copies; MR4: BCR-ABL≤0.01% (IS), with > 10.000 ABL copies. Events: permanent discontinuation of NIL for adverse events, progression to accelerated/blast phase (AP/BP), or death for any reason. ATEs: peripheral arterial obstructive disease (PAOD), acute coronary syndrome, chronic ischemic heart disease, significant carotid stenosis and ischemic stroke.

Results.In the 89 elderly patients, the median age was 71 years; 22% were at high Sokal risk. Molecular response rates were as follows: BCR-ABL/ABL <10% at 3 months, 89%; MMR at 12 months, 54%; MR4 at 12 months, 25%; cumulative incidence of MR4 by 5 years, 75%. These response rates were similar to those observed in patients < 65 years (all p >0.05). Overall, events leading to permanent discontinuation of NIL were observed in 38% of elderly patients, a proportion significantly higher than that of patients < 65 years (23%; p=0.005). ATEs occurred in 15 (16.9%) elderly patients, and were the reason of permanent NIL discontinuation in 12 (13.4%) of them. As expected, the rate of ATEs in younger patients was significantly lower (5.8%; p=0.0035). Nine (10%) elderly patients died during the follow-up: a patient after progression to AP/BP (the only progression observed in patients ≥ 65 years), while the others died in CP for concomitant diseases / age-related conditions (2 patients had a previous ATE). The 6-year progression-free survival and overall survival in elderly patients were 85%.

CONCLUSION. NIL as first-line treatment of newly diagnosed CP CML patients showed high efficacy in patients ≥ 65 years, with molecular response rates comparable to those observed in younger patients. However, a significantly higher proportion of elderly patients discontinued NIL, partially because of the occurrence of ATEs. Even though a relevant morbidity was associated to some ATEs, they did not significantly impacted on the long-term outcome of elderly patients.

P074

THE HOCT1/ABCB1 POLYMORPHISMS DON'T CONDITION THE CYTOGENETIC AND MOLECULAR RESPONSE OR TOLERABILITY TO FIRST-LINE NILOTINIB IN CHRONIC MYELOID LEUKEMIA PATIENTS

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Introduction. Drug transporters, such as ABCB1, hOCT1, and ABCG2, control both intracellular and systemic plasma concentrations of BCR-ABL inhibitors (TKIs). Our group previously reported that the hOCT1 c.480C>G polymorphism conditions higher rates of adverse events and shorter event-free survival (EFS) for imatinib. Even if nilotinib has less substrate affinity than imatinib for these transmembrane transporters, they could have a role on efficacy and/or toxicity of nilotinib.

Methods. Through an approach similar to the one previously used for Imatinib, we investigated any possible influence of ABCB1 and hOCT1 polymorphisms on the nilotinib efficacy and toxicity in a series of 95 CML patients receiving nilotinib as first-line therapy in 7 Italian Centers. Lack of CCyR at 12 months, stop of treatment for any cause, loss of MR3 or CCyR, appearance of mutations were all included as events in the EFS computation. The following polymorphisms were assessed: hOCT1: rs683369[c.480C>G]; ABCB1: rs1128503 [c.1236C>T], rs2032582[c.2677G>T/A], and rs1045642[c.3435C>T].

Results. Fifty-five patients were male and 40 female; the median age was 47 years (range, 18-79); Sokal score was low in 30%, intermediate in 40%, and high in 30% of cases; EUTOS was high in 30%. Efficacy: 96% of patients were in CHR at 3 months; 74% achieved the EMR, 94% the CCyR at 6 months, 95% the CCyR, and 75% the MR3 at 12 months. With a median follow-up of 42

months, 76% achieved a deep molecular response, and only 2.5% failed the MR3. All patients are still alive; with a median time of 49 months, 24% of patients stopped nilotinib, and 21% received <600 mg/day. The 3-year EFS was 77%. Patients were grouped according to the absence or the presence (heterozygous or polymorphic homozygous) of at least one polymorphic allele. We found that ABCB1 and hOCT1 polymorphisms did not condition the achievement of CHR, CCyR, MR3, or DMR. Time to the MR3 was 6 months longer for patients with polymorphic hOCT1 (13 months vs 7; p=0.01); nevertheless, EFS did not differ between the same groups. Toxicities were observed in 43% of cases, with 40% of them of grade 3 WHO; ABCB1 and hOCT1 polymorphisms did not condition toxicities or nilotinib discontinuation. Interestingly, time to the hematological toxicity was longer for heterozygous or polymorphic homozygous than for wild-type cases (19.0 vs 4.4 months; p=0.05).

Conclusions. Our previous studies showed that polymorphic hOCT1 [c.480C>G] did negatively impact on CCyR and EFS during imatinib treatment, and that the combination of ABCB1 and hOCT1 polymorphisms conditioned higher levels of toxicity. On the contrary, the present study demonstrated that the same polymorphisms did not condition efficacy or toxicity when patients received nilotinib as first-line treatment, hence suggesting that the high efficacy of this second-generation TKI confirmed also in our series is independent from the transmembrane transporters.

P075

A RARE CASE OF CHRONIC MYELOPROLIFERATIVE SYNDROME WITH BOTH JAK2V617F AND BCR/ABL E19-A2 MUTATIONS

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Introduction: Myeloproliferative neoplasms (MPNs) are categorized by the 2016 WHO into two distinct groups of diseases, on the basis of BCR/ABL translocation occurrence, as either Philadelphia-positive Chronic Myeloid Leukemia or Philadelphia-negative MPNs (Ph-MPNs). Ph-MPNs include policytemia vera (PV), essential thrombocitemia (ET) and primary myelofibrosis (PMF), and they are associated to the occurrence of the JAK2V617F, CALR and MPL driver mutations. These are distinct diseases in which the coexistence of a JAK2V617F mutation and BCR-ABL is very uncommon. Herein we report a case of a patient with a MPN co-expressing both the BCR-ABL and JAK2V617F. The rarity of this case was further underlined by the detection of the unusual BCR/ABL p230 fusion protein.

Methods: A 77 year old male patient was diagnosed in 2006 a MPN type ET based on thrombocytosis and bone marrow histology, and treated with Hydroxyurea (HU) since then. In 2013 the PCR analyses showed that the patient was positive for JAK2 V617F mutation. In December 2015 came to our observation for worsening splenomegaly, hyperleukocytosis (WBC:174√ó109/L) and anemia (Hb:8.8 g/dL) unresponsive to HU therapy. Since the peripheral blood smear showed increased basophils, bands and several immature myeloid cells we sought the presence of the BCR/ABL transcript.

Results: The reverse-transcriptase PCR analysis detected the BCR/ABL e19-a2 transcript (p230), confirmed by the FISH analysis, and a diagnosis of accelerate phase CML was made. Is ongoing the analysis of the two mutations on the 2006 BM biopsy. The presence of point mutations of the kinase domain of BCR/ABL gene was excluded by a mutational analysis by Sanger sequencing. Therefore the patient started treatment with the second generation TKI Dasatinib.

Conclusions: The present case and others rare cases reported in literature underline several pathogenetic and diagnostic issues. It would be interesting to clarify whether, a subclone of BCR/ABL mutant stem cell, emerged from a previous JAK2V617F mutated clone or vice versa. Moreover, taking into account the possibility of the coexistence of two mutations, as reported by an Italian group in 2.55% of CML cases, it would be necessary to clarify if both mutations should be detected at the moment of the MPN diagnosis, or most likely, in all MPN cases presenting intermediate clinical features between CML and Ph-MPNs, either at the diagnosis or during the follow-up. This issue is made more compelling by the current availability of target therapies for both CML and PMF. Lastly, it should be noticed that, among the uncommon occurrence of MPN co-expressing the two mutations, our present finding is the second case reported in the literature in which the equally rare expression of the BCR/ABL p230 is associated to the JAK2V617F alteration. The rarity of these observations suggests a causal linkage between the two events. Systematic studies would be valuable in order to elucidate all these issues.

P076

PROTEOMIC PROFILE CORRELATES TO MOLECULAR RESPONSE (MR) IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA (CML)

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Introduction: Very few data are available about the serum protein expression of patients with CML.The aim of our pilot study was to evaluate a correlation between depth of MR and proteomic profile of CML patients,and if possible to find novel potential biomarkers complementary to currently existing proven tools to monitor therapy response.

Methods.Thirty sera from peripheral blood (PB) were sampled from 8 patients in MR1 response,6 in MR2,11 in MR3,and 5 in MR4. For 11 patients serum from bone marrow was also available:in particular 3 were sampled from patients in MR1,3 in MR2,4 in MR3,1 in MR4. Samples were divided into 2 groups according to the achievement of MR3: Group A,which comprised 14 samples from patients with a MR lower than MR3,and Group B, including 16 samples from patients who experienced a MR greater than or equal to MR3.The association of proteomic profile with MR was investigated using the SELDI ToF Mass Spectrometry platform. Expression Differences Mapping analysis was applied in order to generate a cluster peaks list,which describes how a singular peak is expressed in the specimen spectrum. Finally,with the aim to give a preliminary identity to the most differentially expressed peaks,an in silico identification was attempted using the Mascot database search available at www.matrixscience.com.

Results. Comparing PB sera from groups A and B, only a peak at 5075 Da had a p-value=0.05.Comparing sera from patients with different MR(MR1 vs MR2 vs MR3 vs MR4), statistical analysis found two features at 11092 Da and once again that at 5075 Da as differentially expressed and statistically significant (P value=0.0034 and 0.0084 respectively) between MR1 and MR4. In particular the peptide at 11092 Da was overexpressed in MR4 patients. Also the peak at 5075 Da was highlighted as statistically significant,but its significance was less important than that of 11092 Da due to a slightly

worse sensitivity.Regarding the two peaks (5075 Da and 11072 Da) we hypothesized that they were closely related,probably as a fragmentation product.With the aim to quickly give a preliminary identity to the peptide,we attempted an "in silico" mass peptide finger print using the open-source tool available at www.matrixscience.com.The bioinformatic tool identified the peptide with a high probability score, as a truncated part of a larger nuclear protein ZMIZ2.It would interact with the Wnt/β-catenin pathway participating in its activation.As suggested by data from literature, we can try to hypothesize that,unlike non responders,in the serum of responsive patients a part of ZMIZ2 is present,as a consequence of the degradation of the entire protein;this finding results in an under-regulation of the Wnt/β-catenin pathway with consequently a better response.

Conclusions: These preliminary data suggest that the peptide at 11092 Da is very closely related to a good response.Our efforts are now oriented to definitively confirming the identity of the peptide and to clarify its role.

P077

SEQUENTIAL ASSESSMENT OF BCR-ABL1 TRANSCRIPT LEVELS BY DIGITAL PCR (DPCR) IN PH+ CML PATIENTS TREATED WITH TIROSIN KINASE INHIBITORS (TKIS): A COMPARATIVE ANALYSIS BETWEEN DPCR AND QPCR

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Digital PCR (dPCR), by giving an absolute quantification of BCR-ABL1 copies/ µl, is expected to be more sensitive and accurate than qPCR in the assessment of Minimal Residual Disease (MRD) in CML patients (pts). In this study, we aim to comparatively analyse in a sequential manner the BCR-ABL1 transcript levels by dPCR and qPCR in 10 CML pts with MMR (MR3.0) or DMR (MR4.0, MR4.5 and MR5.0) after TKIs treatment. For this purpose, samples for dPCR testing, performed on QuantStudio 3D Digital PCR System, were obtained from CML pts, on time-checks planned for MRD monitoring by standard qPCR performed according to the last International Guidelines. Comparative analyses by dPCR and qPCR were performed in 10 cases with at least 3 sequential determinations of BCR-ABL1 transcript levels every 3 months. In 6 out of 10 pts (60%), dPCR data correlated with qPCR values. At the beginning of the study, 3 pts were in MR3.0, 2 in MR4.0 and 1 MR4.5 class and during at least 9 months of monitoring 21 qPCR and dPCR determinations were made. Eight out of 21 dPCR determinations (38%) had an absolute value of BCR-ABL1 > 0.468 copies/ul and 6 out of these 8 determinations (75%) corresponded to MR3.0 by qPCR. Thirteen out of 21 determinations (62%) had an absolute value of BCR-ABL1 < 0.468 copies/ul: 4/21 (31%) and 9/21 (69%) corresponded to MR3.0 and DMR (MR4.0-MR4.5), respectively. Six out of 9 pts (66%) with DMR had a value of BCR-ABL1 < 0.234 copies/ul, while 5 out of 9 (55%) had levels < 0.156 copies/ul. In 4 out of 10 patients (40%), dPCR data did not correlate with qPCR values. At the beginning of the study, 1 patient was in MR4.0, 2 in MR4.5 and 1 in MR5.0 class. Along the follow-up, 15 qPCR and dPCR determinations were made. One out of 15 dPCR determinations (7%) had an absolute value of BCR-ABL1 > 0.468 copies/ul, corresponding to MR4.0, and 14/15 (93%) had an absolute value of BCR-ABL1 < 0.468 copies/ul corresponding to MR4.0 (29%), MR4.5 (57%) and MR5.0 (14%). Ten out of 15 pts (66%) with DMR had a value of BCR-ABL1 < 0.234 copies/ul, while 6 out of 15 (40%) had levels < 0.156 copies/ul. Our preliminary data suggest that dPCR is more accurate and sensitive than qPCR especially in the pts with DMR. A concordance between dPCR and qPCR has been observed mainly in pts with MMR. Instead, a discordant behaviour was observed in the majority of pts with DMR. Of note, all but 2 samples in DMR had an absolute value of BCR-ABL1 < 0.468 copies/ul, previously described by ROC curve as the value below which pts with lower levels of MRD might be dissected (spec.=63%, sens.=84%). Furthermore, in the subset of pts with DMR and absolute value of BCR-ABL1 < 0.468 copies/ul, we found that 60-70% of them had an absolute value of BCR-ABL1 < 0.234 copies/ul, and that 40-55% had an absolute value of BCR-ABL1 < 0.156 copies/ul. These preliminary results suggest that dPCR could be more useful for DMR monitoring and dissecting the best candidate to discontinuation of therapy with TKIs.

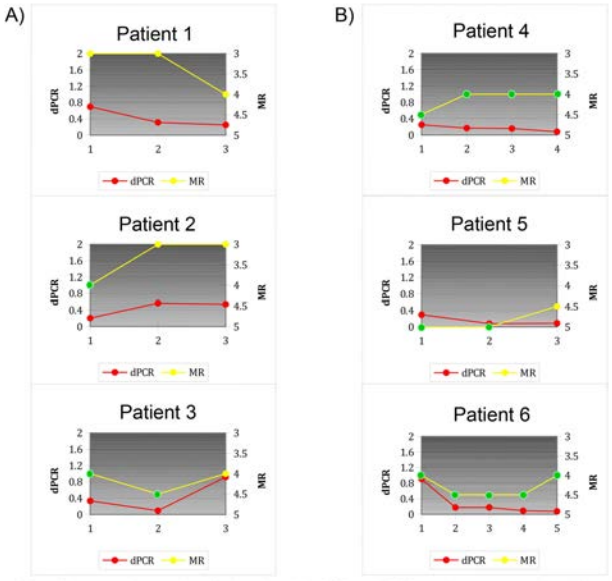

Fig.1. Representative graphs of patients in wich qPCR and dPCR results were concordant (A) and discordant (B). Green dots correspond to undetectable levels of BCR-ABL1 transcript by qPCR

P078

CHRONIC MYELOID LEUKEMIA CELLS SURVIVED AFTER TKI TREATMENT SHOW A SIGNIFICANT INCREASE OF PLURIPOTENT TRANSCRIPTION FACTOR NANOG

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Introduction: Treatment of patients with Chronic Myeloid Leukemia in chronic phase (CML-CP) with tyrosine kinase inhibitors (TKIs) showed a substantially life expectancy improving. However, it is becoming evident that persistent leukemic stem cells can lead to disease relapse at the time of TKI withdrawal in a relevant portion of the patients. Nanog is an essential transcription factor involved in the regulatory networks that are responsible for stemness in embryonic pluripotent stem cells. Functional studies have highlighted that the expression level of Nanog plays crucial role in malignant disease such as tumorigenicity and therapeutic resistance. In our study, we sought to evaluate Nanog role in the regulation of CML cell response to TKI therapy.

Methods: Quantitative RT-PCR (RT-qPCR) for the expression of Nanog was conducted on Ph+ K562 cell line treated with increasing doses of Imatinib (Ima, 0,1-5 μM) or Nilotinib (Nilo, 5-500nM); likewise, western blotting (WB) analysis was conducted for Nanog protein expression. RNA was purified from BM and PB mononuclear cells of 33 CML patients (V0). RT-qPCR for the expression of Nanog and fusion BCR/ABL mRNA was performed and data were normalized by the expression of Gus mRNA (normalized copy number, NCN).

Results: We analysed the Nanog protein expression in K562 Ph+ cells treated with TKI. We observed a significant level of Nanog protein expression in K562 cells treated with 5uM Ima, since 24hours of treatment, with a persistence of expression at least until 72h if compared to untreated control. Moreover, Ima induced the induction of Nanog in a dose dependent manner (range 1-5uM). The RT-qPCR revealed a correlation between Nanog protein and mRNA modulation. Nilo treatment show a different kinetic of Nanog modulation, with a significant upregulation in protein and mRNA expression only after 72h of treatment with 100nM dose. 33 patients enrolled were available for an interim molecular analysis at 24 months: an optimal response (OR) was achieved in 25 patients, a warning response in 5 patients and a failure response in 3 patients. We observed a significant correlation between the expression of Nanog and patients outcome. Indeed, Nanog were significantly downregulated in PB samples at diagnosis of patients with OR compared to patients with warning/failure response (Nanog mRNA: 0.3±0.25 NCN vs 0.6±0.7 NCN, respectively; p=0.05).

Conclusions: These data suggest that Nanog protein expression increases in K562 cells *in vitro* treated with either Imatinib or Nilotinib at a concentration dose compatible with the trough level of the drug at the steady-state when administered at the standard dose (400 mg OD Ima and 300mg BID Nilo). The expression analysis of Nanog in CML-CP patients at baseline, may assist in the early prediction of molecular response in patients treated with Nilo. Further studies are ongoing to functionally evaluate whether Nanog has a role as determinant of TKI resistance and blast transformation in CML.

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P079

TARGETING THE INTERPLAY BETWEEN HSP70 (HEAT SHOCK PROTEIN OF 70KDA) AND HSF1 (HEAT SHOCK FACTOR 1) IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: The search for molecules involved in apoptosis resistance/increased survival of B cells from Chronic Lymphocytic Leukemia (CLL) is still ongoing since this disease remains not completely understood. By a proteomic study, we recently found that the Heat Shock Protein of 70kDa (HSP70), a molecule defined by the literature as "the most cytoprotective protein ever been described", was particularly overexpressed in neoplastic B cells from CLL. HSP70 is induced in response to a wide variety of physiological and environmental insults, thus allowing cells to survive to lethal conditions. HSF1 (heat shock factor 1), is the primary responsible for the transcription of HSP70 being the major regulator of its expression. In response to stress, HSF1 becomes phosphorylated, forms homotrimers, binds DNA and activates heat shock gene transcription. With this background, we are aimed at studying and targeting HSP70 or players related to it (i.e. HSF1) in CLL.

Methods: We treated freshly isolated leukemic B cells from 15 CLL therapy-free patients with: i) Zafirlukast, an oral leukotriene receptor antagonist used to prevent asthma symptoms and acting also as HSP70 inhibitor, at 0, 35, 50 and 70uM for 24h; ii) Fisetin, a dietary flavonoid acting as anti-inflammatory and anti-carcinogen demonstrated to inhibit HSF1 activity through blocking its binding to the HSP70 promoter, at 0, 1, 15, 30uM for 24h. Apoptosis induction in CLL cells was evaluated by Annexin V/Propidium Iodide flow cytometry (FC) test and by the presence of cleaved PARP observed in Western blotting (WB). For Fisetin we also planned an incubation of 30min to verify phosphorylation of Src/Lyn kinase. Phosphorylation of HSF1 at Ser326 and expression levels of HSF1 and HSP70 (HSP72/73) were also analysed.

Results: Considering the pro-survival role played by HSP70 and HSF1, we analysed their inhibition in leukemic cells cultured with Zafirlukast and Fisetin. Both treatments resulted in a dose-dependent cell apoptosis, as assessed by FC and WB. The use of Zafirlukast also induced a reduction in the constitutive form of HSP70 (HSP73). As regard Fisetin, we observed a reduction in phosphorylation of both HSF1 (Ser326) and Lyn (Tyr396). This latter result is intriguing considering that Fisetin was shown to suppress Src activity and Lyn is a crucial Src-family kinase in CLL.

Conclusions: HSP70 overexpression is involved in a diminished response to treatment by promoting the adaptation of tumor cells to changes (i.e. toxic conditions) that are typically created by chemotherapy. HSP70 depletion results in an increased sensitivity to chemotherapy. For this reason, targeting HSP70 in CLL could lead to sensitization of cells to traditional and new anti-leukemic agents. In this context, our results support two additional suggestions: i) the use of Zafirlukast, which is a drug already available for clinical use, in CLL; ii) encourage the targeting of HSF1 in CLL, as the literature has very recently suggested.

P080

REGULATION OF BCR SIGNALLING IN CHRONIC LYMPHOCITIC LEUKEMIA: ROLE OF C-CBL AND CIN85

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Introduction: In B cell chronic lymphocytic leukemia (CLL) little is known about alterations affecting the mechanisms involved in the preservation of homeostasis of Signal Transduction Pathways (STPs). Homeostasis of STPs is maintained by different molecules. c-Cbl (c-Casitas B-lineage lymphoma), an E3 ubiquitin ligase, and CIN85 (Cbl-interacting protein of 85kDa), adapter proteins functioning as docking partners for several signaling proteins, control protein kinase degradation and receptor down-regulation. The accumulation of clonal B lymphocytes in CLL is mostly due to apoptosis resistance but also to proliferative activity. Abnormalities of molecules involved in STPs are connected to CLL pathogenesis and a critical role has already been ascribed to B-cell receptor (BCR)-Lyn axis. We reported that Lyn kinase, is 2.5- up to 5-fold over-expressed in leukemic respect to normal B cells. The evidence that Lyn mRNA level was similar in normal and neoplastic B cells suggested that the anomalous Lyn protein expression was not related to differences in gene transcription and/or mRNA stability but to a deregulation in Lyn turnover. Here, we investigated the expression and the role of c-Cbl and CIN85, that in normal B cells are involved in the ubiquitin-dependent Lyn degradation and in the down-regulation of BCR signaling.

Methods: Blood samples were collected from 15 controls and 30 CLL patients. Untouched peripheral blood B cells were purified using the RosetteSep isolation kit for human B cells. We characterized the protein level of CIN85 and c-Cbl by Western blotting. To evaluate the interaction between CIN85, c-Cbl and Lyn in CLL B cells we performed a co-immunoprecipitation assay, followed by Western blotting analysis, at steady state and after IgM (10 μg/ml) stimulus.

Results: We demonstrated that both CIN85 and c-Cbl are over-expressed ($p<0.001$, Student's t test) and are not constitutively associated in CLL B lymphocytes with respect to normal B cells. We found that in neoplastic B cells CIN85 and c-Cbl did not co-immunoprecipitate with Lyn neither after BCR trigger, suggesting that they are not involved in Lyn turnover. Moreover, we demonstrated that in some neoplastic B cells c-Cbl is constitutively associated with PI3K(p85), influencing BCR signaling. We are currently investigating the basal level of c-Cbl phosphorylation at Y731 in neoplastic B cells.

Conclusions: These preliminary results prompt us to investigate the role of CIN85 and c-Cbl in the development of neoplastic clone. The absence of the association between c-Cbl, CIN85 and Lyn after BCR triggering together with the constitutive association c-Cbl/PI3K(p85) influence cell homeostasis supporting the development of neoplastic clone.

P081

NOCODAZOLE TREATMENT LEADS TO NEOPLASTIC CELL APOPTOSIS IN THE CLL MOUSE MODEL EU-TCL1

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Introduction: CLL is characterized by the accumulation of mature clonal CD19+/CD5+/CD23+ B lymphocytes. Despite their *in vivo* prolonged lifespan, due to defects of cell itself and to microenvironmental stimuli, CLL cells rapidly undergo spontaneous apoptosis *in vitro*. Functional abnormalities of leukemic cells may be related to the microtubular network of cell cytoskeleton. It plays a crucial role in the vital functions of neoplastic cells, including mitosis, motility and cell-cell contact, and for this reason they became an important target in cancer therapies. In particular, tubulin, a cytoskeletal member, is the target of specific drugs, named microtubule inhibitors. Among these, we demonstrated that nocodazole was highly specific in inducing apoptosis of ex vivo leukemic cells from CLL patients, without affecting the viability of either T or MSCs recovered from the same patients. With this as background we were aimed to evaluate nocodazole effectiveness and toxicity in a CLL murine mouse model Eu-TCL1 characterized by high expression of TCL1 protein in B cells leading to the development of a CLL-like lymphoproliferative disease.

Methods. At the age of 13-18 months, a peripheral blood sample from Eu-TCL1 was collected in order to confirm both surface antigen expression and clonality. Cells were stained with antibodies specific for murine CD5, CD19, Ig, Ig, CD3, CD4 and CD8 and evaluated by flow cytometry (FC). Once assessed the presence of disease, 5 mice were treated with nocodazole 10 ng/Kg diluted in a solution of DMSO+10% of polyethylene glycol 350 and 5 control mice with only DMSO+10% of polyethylene glycol 350 (5 days/week for 4 weeks). At the end of treatment, mice are sacrificed and blood, spleen, bone marrow and intraperitoneal wash were collected stained and evaluated by FC. Splenomegaly was also assessed by measurement.

Results. After 4 weeks of therapy, we observed an improvement of treated mice in term of appearance, posture and weight gain with respect to controls. We also demonstrated a decrease in total lymphocyte percentage after nocodazole administration and a higher reduction of pathological B cells (CD19+CD5+) in spleen and bone marrow of treated, with respect to untreated, mice. In human CLL patients after treatment an imbalance of CD4:CD8 ratio was described due to the increase of CD8+ T lymphocytes. Interestingly, in 3 out of 5 mice after nocodazole administration we confirmed a similar expansion of cytotoxic T cells.

Conclusions. The high selectivity of nocodazole and its capability to lead to apoptosis in CLL B cells also in the Eu-TCL1 mouse model, may suggest the use of this inhibitor for designing new therapeutic strategies. Evaluation of further cases could allow us to confirm the pro-apoptotic action on neoplastic B cells and the increase of cytotoxic T cells due to nocodazole treatment.

P082

OFATUMUMAB AND LENALIDOMIDE INHIBIT B-CELL RECEPTOR SIGNALING IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Lenalidomide has clinical activity in chronic lymphocytic leukemia (CLL) exerting pleiotropic activity on the immune system. The combination of lenalidomide with the anti-CD20 monoclonal antibody ofatumumab induces durable responses in patients with relapsed/refractory CLL. In addition to B-cell depletion induced by anti-CD20 antibodies and to immunomodulatory activity of lenalidomide, both drugs directly inhibit survival and proliferation of malignant B cells. The B-cell receptor (BCR) signaling promotes CLL survival and proliferation and is a key determinant of CLL clinical behavior and a therapeutic target. However, the hypothesis that ofatumumab and lenalidomide could target BCR signaling has never been examined in CLL. In this study, we investigated the direct effects of lenalidomide and ofatumumab on BCR signaling in CLL cells.

Methods. The phosphorylation levels of proteins downstream of the BCR signaling, SYK, ERK, PLC-γ, and NF-κB, were analyzed at the single-cell level in 9 CLL cell samples treated in vitro with ofatumumab, lenalidomide or their combination, using phospho-specific flow cytometry. Phosphorylation was measured in the basal condition and following BCR stimulation with anti-IgM.

Results. Ofatumumab and lenalidomide induced different effects in CLL cells from different prognostic subsets defined by the IGHV mutational status. In the IGHV-mutated (M) CLL, ofatumumab, alone or in combination with lenalidomide, induced a significant reduction of SYK and ERK basal phosphorylation. Remarkably, in the same CLL group, lenalidomide significantly inhibited ERK basal phosphorylation. By contrast, in the patient set defined by unmutated IGHV status (UM), ofatumumab and lenalidomide induced no significant changes in basal phosphorylation. BCR stimulation with anti-IgM antibodies induced a signaling response in CLL cells that was statistically significant for ERK, PLC-γ, and NF-κB p65. In the M CLL, ofatumumab, alone or in combination with lenalidomide, significantly inhibited the anti-IgM response of SYK and ERK. Interestingly, lenalidomide significantly reduced the anti-IgM-induced response of SYK. In contrast, in the UM CLL, ofatumumab and lenalidomide induced no significant changes in phosphorylation.

Conclusions. Ofatumumab and lenalidomide can influence the BCR signaling in the basal as well as stimulated states, with differential effects in distinct prognostic subsets of CLL. Overall this study indicates that ofatumumab and lenalidomide inhibit BCR signaling in the M subset of CLL whilst have no significant effect in UM CLL, potentially contributing to the capacity of these drugs to differentially inhibit disease-progression in patients with CLL This study was funded by Fondazione Cassa di Risparmio di Verona, Vicenza, Belluno e Ancona and AIRC (grant #6599). We thank GSK and Celgene for providing ofatumumab and lenalidomide.

P083

GENETIC CHARACTERIZATION OF VARIABLE, DIVERSITY AND JOINING GENES OF IMMUNOGLOBULIN HEAVY CHAIN IN 480 PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: The specific region of B-cell receptor (BCR) that engages close contact with antigens, called CDR3, derives from the recombination of immunoglobulin heavy (IGHV), diversity (IGHD) and joining (IGHJ) genes. The somatic hypermutation process introduces mutation into variable region to increase the BCR affinity for antigens. Given the pivotal role of BCR in chronic lymphocytic leukemia (CLL), we studied the distribution of IGHV, IGHD, IGHJ genes and the length of CDR3 region in patients with CLL.

Method: 621 patients were studied for the BCR genes; 141 were excluded for unproductive rearrangements. For 480 out of 621 (77%) patients data relating to CDR3 length, IGHV, IGHD and IGHJ genes were available and represented our cohort for analyses. Detailed information of RNA extraction, cDNA amplification, purification and sequencing are reported in Visentin A. et al. CLML 2015. IGHV sequences homology ≤98 from the corresponding germline gene, were considered mutated (M-CLL), as opposite to unmutated cases (U-CLL). Chi-square test was used to compare qualitative variables and Kaplan-Meier analyses to estimate overall survival (OS). Analyses of most common stereotyped BCR were made by ARResT.

Results: 26 patients had more than two productive BCR rearrangement and 2 patients had 3. As a consequence, 510 sequences were available and 200 (39%) were classified as U-CLL. 26 subjects had a stereotyped BCR and the most common subsets were #2 (2%) and #1 (1%). The median CDR3 length of our cohort was 13 amino acids (aa). However, we observed that patients belonging to U-CLL have longer CDR3 than M-CLL patients ($p<0.0001$). The average CDR3 lengths for patients with U and M-CLL were 22 and 15aa, respectively. In the cohort, the most common IGHV genes were IGHV1-69 (11%), IGHV3-23 (10%) and IGHV4-34 (7%). The distribution of these genes was skewed ($p<0.0001$); in fact, the most rearranged genes in U-CLL and M-IGHV patients were IGHV1-69 (23% vs 4%) and IGHV3-23 (6% vs 13%), respectively. Considering IGHD genes, the most common were IGHD3-3 (13%), IGHD3-22 (11%) and IGHD3-10 (8%). Also for these genes a non-random distribution among patients with U-CLL and M-CLL ($p<0.0001$) was observed; in fact, IGHD3-3 (24% vs 6%) was the most common gene in the former group and IGHV3-10 in the latter (5 vs 11%). Regarding IGHJ genes, the most common genes were IGHJ4 (47%), IGHJ6 (26%) and IGHJ3 (12%), and their distribution was unbalanced among patients ($p<0.0001$). In fact, IGHJ6 (43% vs 16%) and IGHJ4 (36% vs 55%) were the most common genes in U-CLL and M-CLL, respectively. In addition after a median follow-up of 82 months, the estimated 10-year OS for with CDR3 length <10, 10-20 and >20 aa were 92%, 81% and 57%, respectively (Figure 1, Log-rank test, $p<0.0001$).

Conclusions: We herein provide evidence for a skewed distribution of CDR3 length, IGHV, IGHD and IGHJ among patients with U and M-CLL, which could be related to the different events involved in CLL pathogenesis.

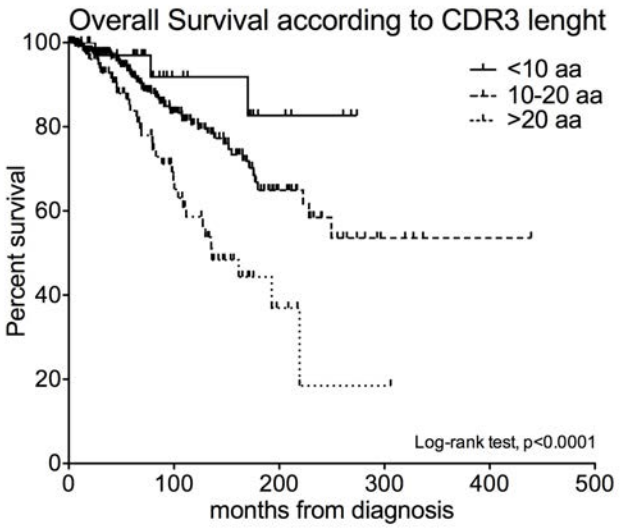

Figure 1.

P084

CLINICAL AND LABORATORY CHARACTERIZATION OF PLATELET DYSFUNCTION DURING IBRUTINIB TREATMENT IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA. MONO-CENTRIC EXPERIENCE

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Introduction: Ibrutinib (IBR) is an inhibitor of Bruton's tyrosine kinase (Btk), approved for treatment na√Øve of chronic lymphocytic leukemia (CLL) with del 17p or TP 53 mutation or for patients (pts) with relapsed/refractory (R/R) disease. Bleeding events were observed in the patients treated with IBR. A defect of platelet function has been hypothesized and inhibition of signaling by glycoprotein VI (GP-VI) has been previously described. IBR associated bleedings and platelet dysfunction may be relevant in CLL pts who are elderly and with comorbidities.

Methods: Nine pts with CLL received IBR at a planned dose of 420 mg/day; 7 were treated with IBR in monotherapy for R/R CLL and 2 in association with monoclonal antibody anti-CD20 for treatment-na√Øve CLL. Median age was 68 years (57-75); 5 pts had unmutated IgVH and 2 had 17p deletion. All pts achieved a partial response; a median follow up was 14 months (range 6-20). Thereafter 2 pts discontinued IBR therapy: 1 for Richter's transformation, 1 underwent allogeneic HSCT. All pts before and after initiation of treatment were studied with light transmission aggregometry (LTA) using platelet-rich-plasma and the following agonists: ADP 2-4 uM, PAR1-AP 25 uM, Collagen 10 ug/mL, arachidonic acid 1 mM, ristocetin 0.6-1.2 mg/mL; measurement of von Willebrand factor(vWF) antigen and ristocetin cofactor activities by chemiluminescent immunoassay.

Results: After a median time of 3 months after IBR treatment we registered only grade I or II bleeding events (bruising, petechiae, conjunctival hemorrhage, rectal bleeding) in 7 pts; no patient needed treatment interruption or dose reduction. Eight pts displayed abnormalities of the aggregation induced by 10 ug/ml collagen after IBR. At these collagen concentration, only significant prolongation of the lag phase was measured (74.6 +/- 23.7 sec vs basal 40.4 +/- 17.2 sec), whereas the maximal aggregation was not impaired (67.9 +/- 21.4% vs basal 85.5+/-5.8%). Interestingly, in 5 pts a significant improvement of the aggregation by 2 uM ADP (91.2 +/- 5.1% vs basal 39.3 +/- 24.6%) and 4 uM ADP (91.6 +/- 2.9% vs basal 65.4 +/- 19.4%) during IBR treatment was reported. On the contrary the aggregation by PAR1-AP, ristocetin and arachidonic acid was not affected under IBR. Finally, in 3/3 pts the vWF antigen and ristocetin cofactor activity were higher at the onset of the disease (169 +/- 38%) and returned to normal values under IBR treatment (111.4 +/- 47%).

Conclusions: IBR treatment in CLL pts causes a mild bleeding phenotype probably due to platelet dysfunction; the bleeding risk was unrelated to platelets count. Collagen induced platelet aggregation resulted impaired while ADP induced aggregation improved upon IBR treatment. Finally the levels of vWF were significantly higher in CLL pts before treatment and normalized during IBR. The assessment of platelet aggregation in IBR treated CLL pts could help to predict and monitor bleeding risk.

P085

INTEGRATED B-CELL RECEPTOR-INDUCED ERK SIGNALING AND SF3B1 GENE MUTATIONS REFINE PROGNOSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Extracellular signal-regulated kinase (ERK) is a major pathway downstream of the B-cell receptor (BCR) and its activation is a significant, independent predictor of shorter clinical progression in chronic lymphocytic leukemia (CLL). Recently identified driver genetic lesions, most likely favored by BCR signals, have reshaped the genetic landscape and added a further level of complexity in CLL. Despite the established driving role of BCR signaling and gene mutations in pathobiology and clinical behavior of CLL, little is known about the clinical influence of integrated BCR responses and genetic alterations. In this study, we investigated the clinical impact of integrated BCR response of ERK and gene mutations in CLL.

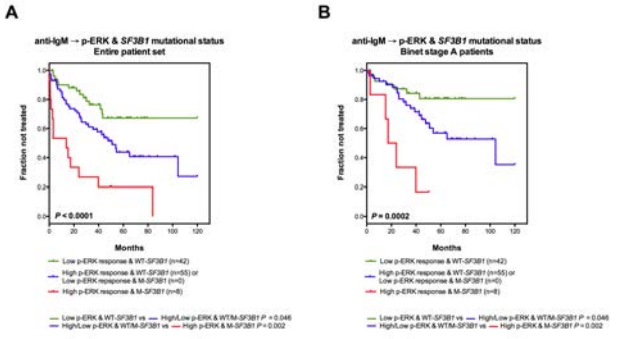

Figure 1: Kaplan-Meier curves of TTFT for subgroups of patinets defined by integrating ERK signaling and *SF3B1* gene mutation.

Methods. Peripheral blood cell samples at diagnosis from 152 CLL patients enrolled in a regional prospective registry (CLL Veneto project) were analyzed in this study. ERK phosphorylation was analyzed using Single Cell Network Profiling (SCNP), a flow cytometry-based assay that allows signaling analysis at the single cell level. NOTCH1, SF3B1, TP53, MYD88, and BIRC3 gene mutations were analyzed by direct DNA sequencing. Univariate and multivariate models for time to first treatment (TTFT) were generated using Cox proportional hazards regression. TTFT curves estimated using the Kaplan-Meier method for the respective groups of patients were compared using the log-rank test.

Results. BCR stimulation with anti-IgM induced a signaling response of ERK (anti-IgM→p-ERK) that was significantly higher in UM subset (P=0.0020), in CD38-positive CLL (P=0.0059), in treated patients (P=0.0003), and in mutated-SF3B1 cells (P=0.0098). Univariate analysis identified increased anti-IgM→p-ERK (P=0.001), UM-IGHV (P<0.0001), positive CD38 (P=0.001), mutated SF3B1 (P<0.0001), as significant, independent predictors of shorter TTFT. In multivariate analysis, only increased anti-IgM→p-ERK (P=0.03) and SF3B1 mutation (P=0.0001) were independent significant parameters of prognosis. Integrating anti-

IgM→p-ERK data and SF3B1 mutations stratified patients in three independent prognostic categories (P<0.0001) and identified an intermediate-risk group that included patients with low p-ERK and mutated SF3B1 or high p-ERK and wild-type SF3B1 (Figure 1A). Consistent with the results in the whole patient set, integrating anti-IgM→p-ERK and SF3B1 mutation in an unselected group of Binet stage A patients (n=110) identified an intermediate-risk group including patients with low p-ERK and mutated SF3B1 or high p-ERK and wt-SF3B1 (P=0.0002) (Figure 1B).

Conclusions. These data assess the prognostic value of ERK signaling in response to BCR stimulation in CLL. Remarkably, they reveal that integrating ERK signaling and SF3B1 gene mutations identifies a category of patient with an intermediate risk of disease progression and refines prognosis in CLL.

P086

BORTEZOMIB EFFECTS ON JAK/STAT PATHWAY IN LARGE GRANULAR LYMPHOCYTE LEUKEMIA

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Introduction: Large Granular Lymphocyte Leukemia (LGLL) is a lymphoproliferative disorder characterized by the clonal expansion of Large Granular Lymphocytes (LGLs) with cytotoxic activity. LGLs proliferation is maintained through the activation of several survival signaling pathways, particularly the JAK/STAT axis. STAT3 activation, mediated by pro-inflammatory citokines and/or activating STAT3 mutations, promotes LGLs survival by inducing the transcription of several anti-apoptotic genes. Nowadays, LGLL therapy is based on immunosuppressive drugs showing however only a partial efficacy (ORR=50-60%). Bortezomib (Bz), a proteasome inhibitor used in Multiple Myeloma therapy, is not used for LGLL therapy, but the rare co-association of MM with LGLL described in literature offered the opportunity to retrospectively investigate the *in vivo* effect of this drug on leukemic LGLs, with promising evidence about its efficacy in reducing the LGL clone The aim of this study is to investigate the *in vitro* effects of Bz on PBMCs from LGLL patients, in order to understand its molecular mechanism of action on JAK/STAT pathway.

Methods: PBMCs of 20 LGLL patients, isolated through Ficoll density centrifugation, were cultured and treated with Bz (5.2 nM) for 24 or 48 hours. Cell apoptosis was evaluated by Flow cytometry, using Annexin V staining and protein expression by western blot (WB) assays.

Results: In order to investigate Bz effects on JAK/STAT pathway, we performed WB analysis to evaluate STAT3 activation by measuring pSTAT3 Tyr705. Our results showed that pSTAT3 Tyr705 levels strongly decreased in Bz-treated condition, both in STAT3 wild-type and mutated patients. This pattern strictly differed from untreated PBMCs, in which STAT3 remained activated. By WB assays, we analyzed the expression of STAT3 downstream targets, focusing on the anti-apoptotic MCL-1 and BCL-2 gene expression. Our data demonstrated a significant reduction of the expression levels of both these genes following Bz treatment (p<0.01). The Bz effect on PBMCs survival was then analyzed by Annexin V staining. Our data showed a significant time-dependent increase of LGLs and monocytes apoptosis after Bz treatment, as compared to control. Interestingly, when purified LGLs were cultured at the same conditions, they were not affected by Bz, suggesting an indirect mechanism of action of this drug, likely correlated to changes in the microenviroment. Finally, to get insights on how monocytes apoptosis could affect LGLs survival, we stimulated PBMCs with two pro-inflammatory cytokines produced by monocytes, IL-6 and IL-15, known to play a role in LGLs proliferation and survival through STAT3 activation. Our data demonstrated that Bz is able to reduce pSTAT3 Tyr705 and to extinguish LGLs survival mediated by these two cytokines.

Conclusions: Our results provide evidence that Bortezomib might represent a new therapeutic option to be added to the immunosuppressive drugs already used in LGLL therapy.

P087

TP53 GENE MUTATION ANALYSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA BY NANOPORE MINION SEQUENCING

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Introduction. MinION is a single-molecule nanopore sequencer from Oxford Nanopore Technologies (ONT) connected to a laptop through a USB 3.0 interface. MinION works by connecting two strands of DNA molecule by a hairpin, and sequencing them consecutively. The single strand of DNA passes through biologic nanopores on a chip, where an electric field is applied and electrical signal variations are recorded. DNA bases are then called using a cloud-based software (Metrichor). Adopting a strategy long template PCR based we tested the MinION for TP53 mutation analysis in CLL patients.

Methods. Twelve CLL patients at diagnosis were analyzed by direct Sanger sequencing (peripheral blood) according to the International Agency for Research on Cancer (IARC) protocol. The same samples were also amplified for TP53 gene in a single Long PCR reaction. Amplicons, spanning from exon 2 to exon 11 (7150bp), were then barcoded and loaded on MinION for sequencing according to the ONT Sequencing protocol (SQK-MAP005). PoRe and NanoOK packages were than used to obtain summary information about MinION nanopore runs, coverage and error analysis. Variant analysis was achieved in Galaxy (https://usegalaxy.org/), where we created a complete workflow performing from reads mapping to variant calling and filtering.

Results. We focused our study on selected CLL patients bearing the 17p deletion to increase the chance to detect TP53 gene mutation. Indeed, five of them resulted mutated from Sanger analysis Sequencing from MinION produced 22421 reads (38Mbases total). Among them, 3619 passed quality filter and 2652 had a recognizable barcode. One of the major drawbacks in using MinION, is the high error rate of sequencing data (around 10%). Thus, we tested two in silico methods for reads correction: nanocorrect pipeline and ALEC (Amplicon Long-read Error Correction) python script. Before correction, error analysis against TP53 genomic sequence (reference: NC_000017.10) returned a mean of 15% of error rate per 100bp aligned. After correction the mean error rate dropped to 4-5% Coverage analysis showed that TP53 gene sequence from exon 2 to exon 11, introns included, was entirely and uniformly covered in each patient (ranging from 50x to 200x). All mutations detected with Sanger were also detected from raw reads and ALEC corrected reads. Conversely, nanocorrect corrected reads produced two false negatives.

Conclusion. In summary, we have shown that MinION is suitable for the detection of TP53 gene mutations in CLL. Its low cost (1000$) and ease of use may include it among the tools of choice for TP53 mutation analysis in hematological malignancies, and potentially in other cancers. Currently, it cannot completely substitute Sanger sequencing nor principal NGS technologies, but it can be an effective prescreening strategy. Anyway, the constant and rapid improvements of nanopore technology are promising for an exclusive and convenient use of MinION in this kind of analysis.

P088

TARGETING THE CXCR4 DOWNSTREAM SIGNALLING PATHWAYS TO REVERSE MICROENVIRONMENT PROTECTION IN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Tumor microenvironment protects chronic lymphocytic leukemia (CLL) cells from spontaneous and fludarabine-induced cell death through CXCL12/CXCR4 axis. CXCR4 is a chemokine receptor constitutively expressed on CLL cells, and the binding with its ligand CXCL12 activates Ras/ERK1-2/Akt and RhoA-dependent signalling pathways. We have recently shown that co-culture with stromal cells (SC) induces in CLL cells the activation of Ras/ERK1-2 and RhoA/RhoA kinase signalling, the upregulation of the pro-survival factor Akt, and an increase in the activity of the transcription factor HIF-1α.

The aim of this study was to evaluate the ability of specific inhibitors of CXCR4 downstream signalling pathways to reverse the protection exerted by SC towards spontaneous and fludarabine-induced apoptosis in CLL.

Patient-derived CLL cells were cultured alone or with murine M2-10B4 SC. In selected experiments, recombinant CXCL12 (100 ng/ml), CXCR4 inhibitor AMD3100 (5 μg/mL), fludarabine (F-ara-A, 10 μM), simvastatin (Sim, 1 μM), ERK1-2 kinase inhibitor PD98059 (10 μM), HIF-1α inhibitors YC-1 (10 μM) and BAY87-2243 (1 μM), or idelalisib (10 μM) were added. Ras and RhoA activities were evaluated by pull-down assay and by an ELISA based assay, respectively. ERK1-2, p70 S6K and HIF-1α were evaluated by Western Blot. RhoA kinase, Akt and HIF-1α activities were measured with specific immunoassay kits. CXCL12 was quantified by ELISA. Cell viability was determined by Annexin-V/propidium Iodide assay and flow cytometry analysis.

The SC-induced activation of Ras- and RhoA-dependent signalling pathways observed in CLL cells was reproduced when CLL cells were exposed to recombinant CXCL12, and was completely abrogated by the CXCR4 antagonist AMD3100. These data unveiled the key role of the CXCL12/CXCR4 axis in the SC-induced modulation of Ras- and RhoA-dependent intracellular signalling. We confirmed that blocking the activity of Ras and RhoA with Sim, and targeting ERK1-2 kinases and HIF-1α by specific inhibitors significantly reduced the constitutive activity and the SC-induced upregulation of Ras- and RhoA-dependent signalling in CLL cells. In the SC counterpart, Sim significantly reduced the secretion of CXCL12, which is a known transcriptional target of HIF-1α. Interestingly, the inhibition of CXCR4 downstream pathways at different levels with Sim, PD98059, YC-1 or BAY87-2243 counteracted the SC-mediated protection of CLL cells from spontaneous and fludarabine-induced cytotoxicity. The inhibition of PI3K and downstream Akt with idelalisib reduced HIF-1α expression and hindered the protective effect exerted by SC on CLL cells.

Our data demonstrate that the inhibition of ERK1-2, Akt and HIF-1α in CLL cells counteract the protective effect of SC against spontaneous and fludarabine-induced apoptosis. The targeting of CXCR4 downstream signalling pathways in CLL cells is a promising strategy to overcome fludarabine resistance, a known feature of disease aggressiveness.

P089

DOWNREGULATION OF IFN-γ PRODUCING T CELLS IS ASSOCIATED WITH TH17 AND REGULATORY T CELLS FREQUENCY INCREASE IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Introduction. Chronic lymphocytic leukemia (CLL) is the most frequent leukemia in the Western world and it is characterized by the clonal expansion of CD5 positive B cells. In CLL, different T cells dysfunctions have been described, probably related to the interaction with malignant B cells and potentially influencing the patient's outcome. TH17 and regulatory T cells (Treg) are subpopulations of T lymphocytes which play a key part in inflammatory response and immune tolerance. However, their role in CLL has not yet been fully clarified. In the present study we focused our attention on the importance of the interplay between Tregs, TH17 and immune status in CLL patients.

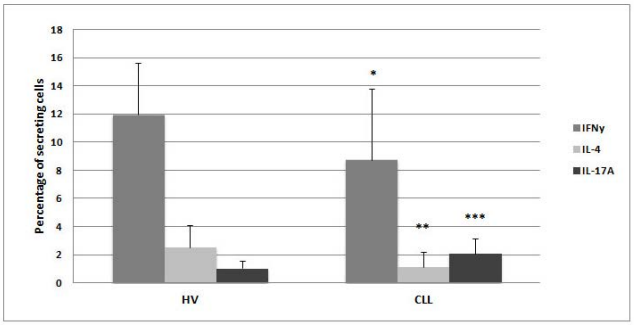

Figure 1.

Methods: After obtaining the patient's informed consent, peripheral blood was collected from 20 newly diagnosed CLL patients without infections and from 20 age-matched healthy volunteers (HV). Mononuclear cells (PBMCs) were separated by density gradient centrifugation and CD4+ cells were isolated by negative immunomagnetic depletion. Cells were cultured in complete medium, primed with IL-6 o/n and with PMA, ionomycin and monensin for 5 h. An unstimulated control was included for each experiment. After stimulation, cells were fixed, permeabilized and immunophenotyped for intracellular IFN-γ, IL-4 and IL-17A expression, using the human TH1/TH2/TH17 phenotyping kit. For cytokine secretion analysis, stimulated CD4+ cells were analyzed using human IL-17 and IL-10 secretion assay detection kits. For Treg analysis, unstimulated PBMCs were stained with anti-CD4 FITC, anti-CD25 APC-Cy7 and then fixed, permeabilized and stained with anti-FoxP3 APC, anti-Tbet PE and anti-RORγt PE. Appropriate isotype controls were included for each sample.

Results: In CLL patients we observed a reduced production of IFN-γ and IL-4, respectively from TH1 and TH2 and an increase of IL-17 from TH17 compared to HV. All the observed differences were statistically significant (Figure 1). We also evaluated the ability of TH17 to produce IFN-γ or secrete IL-10. We reported a statistically significant increase in the frequency of CD4+ IL-17/IFN-γ+ double producing cells in CLL patients compared to HV, whereas the percentage of IL-17+/IL-10+ cells remained unchanged. In addition our data highlighted a significantly higher frequency of CD4+ CD25high FoxP3+ cells (Tregs) in CLL samples, with a meaningful increase in Tbet+ Tregs subpopulation (Th1-like cells). No differences were observed for RORγt+ Tregs (Th17-like cells).

Conclusions: Our results report a down-regulation of IFN-y producing T cells associated to an increased frequency of Tbet+ Tregs and IL-17A/IFN-γ-double producing T cells, probably induced by the interaction with leukemic B cells in order to support the reduced pro-inflammatory response. Starting from these observations it could be interesting to deep investigate the immunological status of CLL patients for improving therapeutic strategies in this disease.

Lymphomas and Multiple Myeloma 2

P090

ENHANCED BEZ235 SOLUBILITY USING OPTIMIZED CALCIUM-CARBONATE NANOCAPSULES IMPROVES THE DRUG EFFICACY IN T LYMPHOMA A CELL LINES

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Introduction. The benefit of nano-sized drug carriers is related to their ability of improving drug solubility, prolong systemic drug half-life, supply means for sustained and environmentally responsive drug release, allow tumor specific delivery, reduce immunogenicity and systemic side effects and deliver simultaneously two or more drugs for combined therapy. Calcium carbonate nanoparticles can be considered an ideal drug carrier due to their excellent biocompatibility and the ability to readily penetrate cancer cells, *in vitro*. Efficient encapsulation of anticancer drugs in nanocolloids and nanocapsules was recently developed by the Nantional Nanotechnology Laboratory of the University of Salento research group. BEZ235 efficiently blocks the dysfunctional activation of the PI3K/mTOR pathway in cellular and *in vivo* settings, thus inhibiting the growth and proliferation of various cancer cells.The very poor solubility in water and ethanol has interfere with future development of the drug beyond phase,ÄäI/Ib clinical trials. In order to overcome the solubility issue BEZ235-loaded nanocapsules were generated by the stepwise adsorption of oppositely charged polyelectrolytes into biocompatible CaCO3 cores and then *in vitro* effects were evaluated. Methods. MTT assay was used to compare the cytotoxicity of free BEZ235, empty nanocapsules and incapsulated BEZ235. A selective and sensitive LC-MS/MS method was developed and validated for the quantification of BEZ235 at different concentrations (0.05microM to 1microM) and time points (2-5-12-24 h) in cell lysates. Cellular extracts of cells treated with empty capsules, free-BEZ235 (1microM) and encapsulated BEZ235 (1microM) for 5, 12 and 24 h were probed with antibodies against PI3K, p-Akt, Akt, p-m-TOR, m-TOR, caspase 3, 8 and 9. In order to track the kinetic of internalization and localization in the cells, the stained FITC nanocapsules were studied by confocal laser scanning microscopy.

Results and Conclusions. The results suggested that nanoencapsulated BEZ235 was extremely efficient compared with free-BEZ235, reaching IC50 (1 microM) just after 5 hours of exposure. Intracellular concentration of BEZ235, quantified by LC-MS/MS method, was consistent with biological results since the internalization kinetic and efficiency was increased by the coating. No cytotoxicity was observed for the empty capsules. In order to confirm that the encapsuled-BEZ235 was still effective on cell apoptosis, we tested free BEZ235 and encapsulated BEZ235 at a concentration of 1 μM in T cell lymphoma cell lines. Encapsulated-BEZ235 induced apoptosis evidenced by the cleavage of caspase 8, 9 and 3 at an earlier time point compared with free BEZ235 and at significantly lower concentration (1microM). Keeping in mind a future clinical application of these capsules, our data can be regarded as a promising new nanotechnology-based strategy to improve the efficacy and bioavailability of old and new drugs.

P091

LENALIDOMIDE TWENTY-FIVE MG EVERY OTHER DAY IN PATIENTS AFFECTED BY MULTIPLE MYELOMA AND RENAL FAILURE

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Lenalidomide is a derivative of Thalidomide, with tumoricidal and immunomodulatory activity. It is available as oral agent, which is effective in the management of newly diagnosed, relapsed or refractory multiple myeloma and as maintenance therapy after autologous stem-cell transplantation.

With normal renal function, Lenalidomide administered orally reaches its maximal plasma concentration after a median time of 0.6-1.5 h, and it is eliminated through glomerular filtration and active tubular secretion in 3 to 4 hours. Serum half life increases up to 9 hours if moderate/severe renal impairment is present (creatinine clearance <50 or <30 mL/min, respectively). In the latter cases a reduction of the daily dose is recommended. However, there is no theoretical assumption that protracting the full standard doses could be equally effective and tolerated in patients requiring reduced doses.

19 patients, 11 female and 8 male, with a median age of 63.3 years (range: 49-81) affected by advanced resistant and progressive multiple myeloma (median number of previous treatment lines: 3, range : 1-5, all including bortezomib) with concomitant renal failure (median calculated creatinine clearance 47.2 ml/min, range : 18-119) were treated with monthly 21-day courses of 25 mg lenalidomide every other day and dexamethasone. Disappearance of urinary light chain and reduction of serum creatinine were detected in 8 patients (42%); 6 patients (31.5%) had a partial response, and 3 of them (16%) were in stable disease, whereas 2 patients (10.5%) had signs of progressive disease. No patient experienced significant myelotoxicity; four patients required red cell transfusions (Grade 3 Anemia). No extra-hematologic toxicity occurred during treatment.

These preliminary observations point to a significant therapeutic effect in more than half of a small population of patients (89.4%) with advanced disease. However, these results have to be validated by controlled studies involving larger number of patients.

P092

BENDAMUSTINE-BRENTUXIMAB COMBINATION IS EFFECTIVE AND HAS A FAVOURABLE TOXICITY PROFILE IN THE TREATMENT OF REFRACTORY AND RELAPSED HODGKIN LYMPHOMA

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The management of patients with refractory or relapsed Hodgkin lymphoma (HL), expecially after autologous stem cell transplantation (ASCT) remains controversial. Bendamustine has demonstrated efficacy in several lymphoproliferative disorders but limited data are available regarding the schedule in patients with HL, in particular its dosage and the possibile combinations for a synergistic effect. Brentuximab Vedotin is a CD30-directed antibody-drug conjugate, currently approved for the treatment of relapsed or refractory HL. The objective of this phase II study was to evaluate efficacy and safety of salvage cytotoxic regimens in patients with refractory and/or relapsed HL. Three different schedules were evaluated.

From May 2011 to September 2015, 24 patients (13 M/11 F) (Table 1) with a median age of 32.5 years (range 16-73) received a salvage regimen after failure of ASCT. Patients were by chance assigned to one of these three arms : standard dose bendamustine (90 mg/sqm) days 1 and 2 plus DHAP schedule (every 4 weeks) x 3 cycles (Arm A), brentuximab single agent 1.8 mg/kg (every 3 weeks) x 4-8 cycles (Arm B), high dose bendamustine (120 mg/sqm) days 1 and 2 plus brentuximab 1.8 mg/kg (day 3) (every 28 days) x 4-6 cycles (Arm C). In arm C, each cycle was repeated every 28 days and growth factor support was sistemically administered, in association with antimicrobial prophylaxis. The treatment efficacy in each group was evaluated according to Revised Response Criteria for Malignant Lymphoma. Any adverse event occurred was recorded and classified for type and grade using NCI-CTCAE criteria (v 4.0). In arm A, (10 patients), the overall response ratio (ORR) was 40%, with 4 (40%) complete remission (CR) and 6 (60%) progressive disease (PD). Extra-hematological toxicities were grade 2 gastrointestinal toxicities in 6 patients (60%) and grade 1 gastrointestinal toxicities in 3 patients (30%), hematological toxicity was grade 3 thrombocytopenia in 4 patients (40%) and bone marrow aplasia in 1 patient (10%). In arm B (6 patients), ORR was 66%, with 3 (50%) CR, 1 (17%) PR and 2 (33%) PD. Extra-hematological toxicities were grade 3 neuropathy in 1 patient (17%), hematological toxicity was grade 2 neutropenia in 2 patients (33%). In arm C (8 patients), ORR was 100%, with 8 CR (100%) and then SCT (4 autologous and 4 haploidentical-SCT) with persistence of complete remission in all patients (median overall survival was 32.2 months (range 27-44) and median progression free survival 15.3 months (range 9-18.4)). Extra-hematological toxicities were increase of transaminase (grade 2) in 2 patients (33%) and citomegalovirus (CMV) reactivation in 2 patients (33%), treated successfully with valgangiclovir. Hematological toxicity was grade 3 thrombocytopenia in 2 patients (33%). High dose bendamustine plus brentuximab has shown significant efficacy in a particular severe setting of heavily pretreated patients, and it could be considered as a bridge to allogenic SCT.

Table 1.

	Arm A (Standard dose bendamustine + DHAP)	Arm B (brentuximab single agent)	Arm C (High dose bendamustine plus brentuximab)
Number of patients	10	6	8
Median age, years	31.5	25.3	35.3
Median number of previous treatments (range)	3 (2-6)	4 (2-7)	6 (2-8)
Overall Response Ratio (ORR)	**40%** 40% CR 60% PD	**66%** 50% CR 17% PR 33% PD	**100%** 100% CR
Treatment following salvage regimen	2 Auto-SCT 1 Haploidentical SCT	2 Haploidentical SCT	4 Auto-SCT 4 Haplo-SCT
Median overall survival, months (range)	22.3 (18-28)	28.1 (23.9-34.5)	32.2 (27-44)
Median progression free survival, months (range)	10.2 (8-19.1)	12.8 (8.5-20.5)	15.3 (9-18.4)
Extrahematological toxicities + grade	60% (6) grade 2 gastrointestinal toxicity 30% (3) grade 1 gastrointestinal toxicity	16% (1) grade 3 neuropatia	33% (2) AST/ALT increase (grade 2) 33% (2) CMV reactivation
Hematological toxicities + grade	40% (4) grade 3 thrombocytopenia 10% (1) bone marrow aplasia	33% (2) grade 2 Neutropenia	33% (2) grade 3 thrombocytopenia

P093

EVALUATION OF FREE LIGHT CHAINS (FLC) IN SERUM AND BONE MARROW IN PATIENTS WITH MULTIPLE MYELOMA

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Background: Monoclonal free light chains (FLCs) dosage is an important tool to evaluate treatment response in patients with Mul-

tiple Myeloma. In this study we evaluated if there is any difference in FLC concentration between bone marrow and serum. Patients and methods: 29 pts with Multiple Myeloma (MM), 6 in remission and 23 with active disease (diagnosis, resistance or recurrence), were studied. Concentration of FLC and K/L ratio were tested in serum (sK/L) and in marrow (mK/L) samples.

Results: All pts with active MM had altered K/L. In 18 pts, the values in serum and marrow are very similar (range ratio mK /L and sK/L: 0.8 - 1.2), instead, in 5 pts different results were observed in the two types of samples: In 2 pts (MM IgG k) mK/L was higher than sK/L (9,79 vs 4,77 e 10,3 vs 3,58). In 2 pts with progressive disease (MM IgG k) sK/L was higher than mK/L (122,86 vs 74,84 e 25,22 vs 14,15). In 1 pt with refractory disease (MM IgG L) sK/L was higher than mK/L (0,13 vs 0,31). In 6 pts with Complete Remission of disease, sK/L was normal; only in 1 pt (not secretory MM) mK/L was slightly higher than sK/L (0,21 vs 0,35)

Conclusions: Evaluation of FLCs in the bone marrow does not seem to provide additional informations in pts with Multiple Myeloma. It remains unclear whether the differences observed in some patients have biological reasons and may, once explained, have a significant impact on the clinical assessment.

P094

LOWER FOXP3+ CELL COUNTS AND HIGHER MIR-144 LEVELS AT DIAGNOSIS PREDICT TIME OF RELAPSE IN FOLLICULAR LYMPHOMA PATIENTS

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Introduction: First line treatment of follicular lymphoma (FL) patients is followed by a variable disease-free time before the disease relapse. No molecular marker enables to predict the risk of relapse of these patients so far.

Methods: We investigated the expression profile of miRNAs in 26 FL tissues(8 grade I, 5 grade II, 9 grade IIIa and 4 grade IIIb) using microarrays containing probes assessing 353 miRNAs and the immunohistochemical expression of Foxp3, Pdcd1 and Cd68. Twelve reactive lymph nodes (LN) were used as reference.

Results: FLs showed overexpression of 17 miRNAs and down-regulation of 12 miRNAs compared to reactive LN. Deregulated miRNAs increased from four (grade I) to 11 (grade IIIa). MiR-342 and miR-370 were identified as T-cell signature in FL according to their correlation with CD3 mRNA level. Among 21 treated FL patients with an average follow-up of 13.5 years, 8 cases relapsed. Neither Cd68+ nor Pdcd1+ cell counts were associated with relapse of FL. Increased probability of relapse was a feature of FL with lower total Foxp3+ cells (P=0.05). The expression of 25 miRNAs discriminated relapsed from not relapsed FL; among these, miR-144 level correlated inversely with total Foxp3+ cells counts. Remarkably, miR-144 expression level was higher than the median value in 8 of 9 relapsed as compared to not relapsed FLs, thus predicting the risk of relapse (P=3x10^-5).

Conclusions: Our data suggest that T-cell-mediated immune response and miRNA expression changes are potential prognostic markers in FL. In particular, the combined measurement of Foxp3+ cell count and miR-144 level at diagnosis appears promising in discriminating drug resistant/refractory FL cases.

P095

A CASE OF PROMPT DIAGNOSIS OF PRIMARY MEDIASTINAL LARGE B CELL LYMPHOMA BY FLOW CYTOMETRY PERFORMED ON TRANSBRONCHIAL BIOPSY

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Backgrounds: We describe a prompt flow cytometric diagnosis of Non Hodgkin B cell Lymphoma in a young 32 years old female patient. The patient was admitted at the thoracic surgery unit of our hospital because of the presence of a bulky mass in the anterior mediastinum. At clinical evaluation signs and symptoms were breathing difficulty, upper body edema with dilated veins of the anterior wall of chest and neck, dysphagia and nocturnal sweating. Because of the presence of bilateral breast prosthesis the surgeon equipment decided to not perform a diagnostic mediastinotomy. As an alternative to surgery a bronchoscopy was proposed to obtain a bronchoalveolar lavage and a transbronchial specimen for diagnostic purpose. Both biological materials were sent to our lab in order to evaluate a possible presence of lymphoproliferative disorder and a B cell clonal restriction.

Methods: Immunophenotypic analysis was realized on bronchoalveolar lavage and on transbronchial biopsy of the mediastinal mass. We performed a lyse and wash staining method with eight multicolor combinations. We acquired the stained cells using a FACSCantoII cytometer (BD Beckton Dikinson). BDFACSDiva software was used for flow cytometric analysis. A positive reaction was defined when ≥20% cell population expressed a specific antigen. Cell suspension was obtained by means of a mechanical tissue disaggregation of the transbronchial biopsy by Medimachine.

Results: The immunophenotypic examination of bronchoalveolar lavage revealed a B cell population CD19+ with positivity for CD45 dim expression, FMC7, CD20 bright expression and with negativity for CD10, CD34, CD58, CD23, CD43, CD79b, CD5. The cells were large in size and with high cellular complexity. The study of Kappa and Lambda chain surface expression was also evaluated in the B cell population, showing 43% without light chain expression, 30% with Kappa positivity and 20% with Lambda positivity, without clonal surface light chain restriction. The same immunophenotypic pattern was also demonstrated in the cell suspension obtained by disaggregation of transbronchial biopsy.

Conclusions: The peculiar phenotype expected in Large B cell mediastinal lymphomas, which differs considerably from that of other B cell lymphomas, is typically characterized by the absence of light chain surface expression. Therefore we concluded for a diagnosis of Primary mediastinal Large B cell Lymphoma. The patient promptly started chemio-immunotherapic treatment regimen according to R-CHOP schedule, showing a rapid improvement of clinical signs and symptoms. The diagnosis was later confirmed by histological examination. Hence, we concluded that flow cytometry could be a useful and rapid diagnostic assay with a supportive role to surgery and histologic diagnosis in selected patients.

P096

TRANSGASTRIC ENDOSCOPIC ULTRASOUND-GUIDED FINE NEEDLE ASPIRATION BIOPSY OF ISOLATED SPLENIC LESIONS FOR LYMPHOMA DIAGNOSIS

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Introduction: Isolated splenic lesions are relatively infrequent, but represent a diagnostic challenge, as tissue sampling for histologic analysis may be difficult, in particular if the size of the lesion is small. Percutaneous image-guided splenic biopsies are rarely performed due to the perceived high risk of hemorrhage that could lead to urgent splenectomy, and diagnostic splenectomy is often preferred as primary diagnostic procedure. Endoscopic ultrasonography(EUS)guided fine needle aspiration (EUS-FNA) biopsy allows sampling of tissue which is adjacent to the stomach wall. EUS provides a good imaging of the spleen through the gastric wall. There have been only few cases of successful histopathologic diagnoses by EUS-guided splenic biopsies reported in the literature. We report our experience with trans-gastric EUS-FNA in the investigation of patients with isolated splenic lesions suspicious for lymphoma. We retrospectively assessed the diagnostic performance and safety of EUS-FNA to elucidate the tissue diagnosis of splenic abnormalities suspicious for lymphoma.

Methods: Between 2009 and 2016, we evaluated ten consecutive patients with splenic lesions detected by Computed Axial Tomography (CT), Positron Emission Tomography (PET) and Ultrasonography (US) in our Institution. Transgastric EUS-FNA biopsy was performed by a linear echo-endoscope and 19/20 gauge needles. Patients were admitted to the hospital to guarantee an overnight monitoring after the procedure. Platelet counts were in the normal range and INR was <1.5.

Results: The age of the patients was 40-83 years (average 57.7 years); six patients were male and four female. All patients presented focal splenic lesions that varied in size from 17 to 100 mm (average 41 mm). Bone marrow biopsy was negative for lymphoma localization. There was no evidence of bleeding or other complications related to the procedure after splenic EUS-FNA biopsy. In eight patients tissue sampling was sufficient for a pathological diagnosis (80% sensitivity). Diagnoses were Hodgkin Lymphoma in two patients, and Diffuse Large B-cell Lymphoma (DLBCL) in six patients. Only two patients required further diagnostic procedures, including laparoscopic spleen biopsy in one patient and splenectomy in one patient. In both cases final diagnosis was DLBCL.

Conclusions: Transgastric EUS-FNA biopsy of isolated splenic lesions has been shown to be a safe diagnostic procedure with high sensitivity for the diagnosis of lymphoma, reducing the need for splenectomy.

P097

BENDAMUSTINE-BORTEZOMIB-DEXAMETHASONE IN RELAPSED MULTIPLE MYELOMA

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Background. Bendamustine is a multifunctional alkylating agent with single agent activity in myeloma. Increasing evidence from clinical studies demonstrates the efficacy of bendamustine as first-line treatment for multiple myeloma, and in heavily pre-treated relapsed/refractory patients.

Aim. The aim of this retrospective study was to evaluate the efficacy and toxicity profile of bendamustine, bortezomib, and dexamethasone (BBD) combination treatment in patients with relapsed multiple myeloma (MM)

Methods. We report the treatment outcome for 8 (6 male, 2 female; median age: 72y, range: 66-78) relapsed MM patients. Treatment consisted of bendamustine (90 mg/m^2 I.V. on days 1, 2), bortezomib (1.3 mg/m^2 S.C. on days 1, 8, 15, 22) and dexamethasone (40 mg I.V. on days 1, 8, 15, 22) for all patients. Each cycle was 4 weeks, and median number of treatment cycles was 3. Patients had a median of three prior lines of therapy. All patients had received prior bortezomib and/or lenalidomide. One patient received peripheral blood stem cell transplant.

Results. The clinical stage (remission, progression or stable disease) was defined with clinical re-evaluation after chemotherapy and re-staging 6 months after end of therapy. 6 patients had a confirmed partial response or better, including 2 patients with a very good partial response. 1 patient showed a progression disease. Grade 3 hematological effects and grade 3/4 non-hematological effects occurred in 4 and 3 patients, respectively. Most pronounced hematological adverse event was leukopenia.

Conclusions. Bendamustine in combination with Bortezomib and Dexamethasone demonstrates an excellent effectiveness in previously treated MM patients, with an acceptable toxicity profile. These agents, when compared to traditional chemotherapeutic agents, may lead in the future to higher responses, longer remissions and better quality of life for patients with MM.

Stem Cells & Growth Factors

P098

USE OF COMBINED ORAL ADMINISTRATION OF ANALGESIA AND ANXIOLYSIS FOR PAIN ASSOCIATED WITH BONE MARROW ASPIRATION AND BIOPSY

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Bone marrow aspiration and biopsy (BMB) is central to the diagnosis and management of many haematological disorders and is a safe procedure associated with low morbidity and mortality. For adults, the infiltration of local anesthesia at the biopsy site has been used as the principal form of analgesia for BMBA. Unfortunately pain relief is often incomplete especially during aspiration of the bone marrow. In addition, pain is likely to contribute to the anxiety the patient may already be experiencing. In this study we assessed an oral administration of analgesia (fentanil - ACTIQ) and anxiolysis (midazolam). 107 consecutive ambulatory adult patients referred for bone marrow examination were enrolled. Informed consent for the procedure was obtained from all patients. All patients received local anaesthesia (LA) with 10 mL of injected 2% lignocaine, but 52 patients received LA alone (group A) and 55 patients LA plus 5 mg midazolam (oral administration) and 200 mcg of Fentanyl trans-mucoso (group B), 30 min before the procedure. The pain level was assessed with the Numeric Rating Scale which distinguishes ten levels of pain, from 0 to 10 in five times of procedure (baseline, start LA T1, aspiration T2a, biopsy T2b, five minutes after the end of the procedure T3). At the end, all were given a questionnaire about efficacy, satisfaction, comfort with three levels (1/low - 2/medium - 3/high).

This medium values were found : at time T1 the medium level of pain was 0.87 for the group A vs 0.88 of group B, at time T2a 3,63 group A vs 3,54 group B, at time T2b 4,63 group A vs 4 group B ($p<0,05$), time T3 0,41 group A vs 0,16 group B ($p<0.05$). In addition 21 Patients, who have already undergone the procedure without sedoanalgesia, saw to prefer the new medication. Our preliminary results seem interesting because underline the different subjective perception of pain in the two groups and especially show a main level of satisfaction and comfortable in our patients undergone medication with sedoanalgesia and a lower level of anxiety in view of a possible repeat of examination.

P099

MYELODYSPLASTIC SYNDROMES AND ERYTHROPOIESIS STIMULATING AGENTS (ESAS) : EVALUATION OF ERYTHROPOIETIC ASPECTS AND ANALYSIS OF RESPONSE

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ESAs are the frontline treatment in low-risk anemic MDS patients and an employment of this therapy in the earlier stage of the disease can delay the need for RBC transfusion, hypothetically by slowing the disease course. It's matter of debate whether the clinical response is a result of proliferation and maturation of the dysplastic clone or stimulation of residual normal erythropoiesis by ESAs. Macrocytosis is one of the cytological hallmarks of dyserithropoiesis in MDS : an analysis of the erythropoietic response to ESAs therapy in a cohort of anemic non trasfusion-dependent MDS patients, enrolled in a retrospective register, RECAMDS, subgroup of Italian register, was performed. 137 patients, treated with standard-dose ESAs, have been retrospectively analyzed. Data analysis was performed, according to IWG2006, at the baseline, after 3 and 6 months of continuous treatment, with a subanalysis of the patients according to WHO and R-IPSS risk stratification. ESAs were started at mean Hb concentration of 9.5 g/dl, mean serum EPO concentration : 41 mU/L, after a mean time from diagnosis of 6 months (r.1-118). Overall response rate (ORR) was 83% (114/137), no difference among WHO and IPSS subgroups was found :76% achieved response after 3 months of treatment, while other 7% after 6 months. 2 patients with SD (non responders IWG), in which treatment was continued, achieved response after 9 months. In the macrocytic-responders group 87% exhibits again macrocytosis after 3 months, while 13% become normocytic. In the normocytic-responders group 92% exhibits again normocytosis, while 4/52 (8%) become macrocytic : in these 4 patients after 3 months there was a contemporary worsening in neutropenia and thrombocytopenia, with transfusion-dependence, regarded as first signs of progression of disease. Non responders were 23/137 (17%) : in the macrocytic-non responders group 89% exhibit again macrocytosis after 3 months, while 11% become normocytic; in the normocytic group 80% exhibits again macrocytosis, while 20% become normocytic : r.1-23). These preliminary data can suggest that, in the majority of MDS patients responsive to ESAs, the increase of Hb concentration occurs mainly stimulating erythroid production in MDS clones; in the minority of patients probably it happens recruiting residual polyclonal erythropoiesis. It is interesting to note that stimulating effects of ESAs last even when the expression of dysplasia progresses.

P100

ROLE OF THIOTEPA DOSE IN THE CONDITIONING REGIMEN THIOTEPA-BUSULFAN-FLUDARABINE (TBF) FOR HAPLOIDENTICAL TRANSPLANTS

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Introduction: A conditioning regimen including Thiotepa, Busulfan and Fludarabine (TBF) is commonly used for patients with high-risk hematological malignancies undergoing hematopoietic stem cell transplantation (HSCT) from an haploidentical related donor.

Methods: We collected the data of 20 consecutive patients undergoing HSCT from a related haploidentical donor between January 2012 and December 2015. Median age was 60 years (range 23-67), underlying diseases were mainly acute leukemias (75%) and lymphomas (10%); 65% of the patients had an active disease at transplantation, 75% had a HCT-CI ≥ 3. The TBF regimen consisted of Busulfan 3,2 mg/kg for 2 days and Fludarabine 50mg/mq for 3 days in all patients; 9 patients (45%) received Thiotepa 5 mg/kg for 2 days (Thio-10 group) and 11 patients (55%) received Thiotepa 5 mg/kg for 1 day (Thio-5 group). The Thiotepa dose was decided by the physician according patient age and frailty. GVHD prophylaxis was based on Cyclosporine, Mycofenolate-mofetil and post-transplant 100 mg/kg Cyclophosphamide. Patients received a median dose of CD34 positive cells of $2.54x10^6$/Kg from bone marrow.

Results: We did not observe any significant difference in patient age, HCT-CI score, type and pre-transplant status of the underlying hematological disease between Thio-5 group and Thio-10 group. Median dose of infused CD 34+ cells was comparable between the two groups ($2.73x10^6$ in the thio-10 group vs $2.38x10^6$ in the thio-5 group, p=0.47). However, neutrophil engrafment was significantly slower in Thio-5 group in comparison with Thio-10 group [median time to neutrophils $0.5x10^9$/L was 19,2 days vs 17,1 days(p=0.08) ; median time to neutrophils > $1x10^9$/L was 22.2 days vs 17.8 days (p=0.009)]. Median time to platelets > $20x10^9$/L and >$50x10^9$/L was respectively 27.6 days and 32.3 days in the Thio-10 group and 30.5 and 44 days in the Thio-5 group (p=0.59, p=0.30). Median time of

hospitalization was 47 days in the Thio-10 group and 54 in the Thio-5 group (p=0.03). Platelets , red blood cells transfusion units and G-CSF administrations were significantly higher in the thio-5 group (p=0.018 , 0.001 and 0.05 respectively). No differences were found regarding the incidence of bacterial infections, Citomegalovirus reactivation or probable/proven invasive mycoses. Transplant related mortality (TRM) was 27% in the Thio-10 group and 33% in the Thio-5 group (p=0.99).

Conclusions: In our experience, TBF conditioning regimen with 10 mg/Kg Thiotepa had a faster hematological recovery, resulting in a reduction of the days of hospitalization and transfusional support in comparison with 5 mg/Kg Thiotepa. No significant increase of toxicity, infective complications or TRM was observed, suggesting that TBF with 10 mg/Kg thiotepa could be considered a safe conditioning regimen before haploidentical stem cell transplantation even in elderly and frail patients.

P101

EFFICACY OF BIOSIMILAR FILGRASTIM (ZARZIO®) FOR AUTOLOGOUS PERIPHERAL BLOOD STEM CELL MOBILIZATION IN ADULTS AFFECTED WITH ACUTE MYELOID LEUKEMIA: A SINGLE CENTER RETROSPECTIVE STUDY

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Introduction: Biosimilars filgrastim demonstrated similar efficacy and safety, compared with originator (Neupogen®, Amgen), in peripheral blood stem cell (PBSC) mobilization in either patients with hematologic malignancies, mainly lymphoma and myeloma, or healthy donors. Hematopoietic stem cell transplantation (HSCT) outcomes were also equivalent. However, information on acute myeloid leukemia (AML) patients undergoing PBSC mobilization with biosimilars is so far restricted to anecdotal cases.

Methods: Over a 10-year period, we observed 88 consecutive adult patients affected with AML (41 F/47 M; median age 53 years, range 21-60). Patients who obtained CR after remission induction chemotherapy underwent consolidation treatment (Ara-C 500 mg/m^2 every 12 h days 1-6 + daunorubicin 50 mg/m^2 days 4-6) with planned harvest of autologous PBSC after mobilization with filgrastim 10 mcg/kg/day s.c. since day +20. We retrospectively compared variables of PBSC mobilization of patients who received either Neupogen® between 2006 and August 2011 (cohort A, 33 patients) or Zarzio® (Sandoz Biopharmaceuticals) since September 2011 to February 2016 (cohort B, 26 patients).

Results: After having received a median of 13 (range 3-60) and 9 (range 3-37) filgrastim doses in cohorts A and B, respectively, PBSC mobilization failed in 6/33 (18.2%) and 5/26 (19.2%) patients, respectively. Neutrophil count recovery was documented on day +23 (range 21-41) and +22 (range 21-26) after treatment with Neupogen® and Zarzio®, respectively. The median peak of circulating CD34+ cells/µl (16, range 0.2-830 in cohort A vs 27.5, range 0.8-176 in cohort B) occurred on days +25 (range 21-50) and +24 (range 21-38) after stimulation with Neupogen® and Zarzio®, respectively. Overall, median harvested CD34+ cells x10^6/kg body weight were 4.7 (range 1-36.3) and 4.9 (range 0.8-14), with 23/33 (66.7%) and 19/26 (73.1%) good mobilizer patients, collecting >2x10^6 CD34+ cells/kg in cohorts A and B, respectively. The median number of aphereses was 1 (range 1-3) in both groups. No statistically significant differences were observed between patients who received Neupogen® or Zarzio®, for the key parameters measured for PBSC harvest. Overall, 22 patients finally underwent autologous HSCT, 11 from each group, with reinfusion of 5.5 (range 3-18) and 4.8 (range 2.8-6.4) CD34+ cells/kg in cohorts A and B, respectively. A trend for earlier recovery of platelet count >20x10^9/L was documented for patients previously mobilized with Zarzio®, with median day of recovery +14 (range 11-24) and +12 (range 11-18) in cohorts A and B, respectively (p=0.03).

Conclusions: The efficacy of biosimilar filgrastim Zarzio® is comparable to the originator in terms of kinetics of PBSC mobilization and yield of CD34+ cells harvest also in the challenging setting of AML patients, in whom adequate PBSC collection is reported to fail in at least 25% of cases. Perspective randomized studies are warranted to further confirm these results.

P102

DONOR-SPECIFIC ANTI-HLA ANTIBODIES AND PRIMARY GRAFT FAILURE RISK IN MISMATCHED HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Introduction. Detection of donor-specific anti-HLA antibodies (DSA) has been reported to be associated with graft failure (GF) in mismatched HSCT, but their frequency and their clinical impact remain unclear.

Methods. We prospectively evaluated the presence of DSAs, using Solid Phase system, Luminex commercial kits (Labscreen Mixed and Single Antigen class I and II, One Lambda), in adult patients undergoing unmanipulated unrelated mismatched HSCT (USCT) and unmanipulated haploidentical SCT (HaploSCT). DSAs binding level was expressed as mean fluorescence intensity (MFI).

Results. Nineteen consecutive patients were analyzed: 8/19 (42.0%) underwent one HLA minor or major mismatch USCT and 11/19 (58.0%) HaploSCT. Patients were affected by AML (9 pts), ALL (3 pts), HL (2 pts), MM (2 pts), MDS (1 pts) and CML (2 pts). USCT conditioning was Busulfan or Fludarabine-based and GvHD prophylaxis Cyclosporine and Methotrexate. HaploSCT conditioning regimen was based on Thiotepa, Busulfan and Fludarabine and GvHD prophylaxis on Cyclosporine, Mycophenolate and post-transplant Cyclophosphamide. DSA were detected in 3 patients (16%): 2 patients underwent HaploSCT and 1 USCT, without receiving desensitization treatment before transplant. All of them failed to obtain allogeneic engrafment. One patient (HL, haploidentical donor) had an autologous engraftment and died of progressive disease 9 months after HSCT. This patient had DSA against HLA-Cw10 (MFI 22400). Primary GF occurred in the other DSA-positive patients. The first one (MM, unrelated donor) died of cerebral hemorrhage on day 28. This patient had DSA against HLA-A2 (MFI 10500) and also anti-HPA1a antibodies associated with platelet transfusion refractoriness. The second patient (AML, haploidentical donor) had DSA against HLA-B50 (MFI 900) and anti-HPA1a antibodies. Patient experienced primary GF with increasing titles of DSA (maximum MFI 10500); so, on day 38, a second transplant from the same donor was performed. Conditioning regimen was: Fludarabine and Treosulfan, GvHD prophylaxis was: Sirolimus, Mycophenolate and ATG. Before reinfusion, the patient was also treated with Rituximab (375 mg/sm), plasma exchange and intravenous immunoglobulin (1 g/kg) to decrease DSA levels. Stem cells source was peripheral blood without manip-

ulation. A progressive decrease in DSA was documented (up to MFI ≤ 200) (Figure 1). On day +12 patient achieved neutrophil count over 500/µl and on day 23 platelet count over 20000/µL. Moreover, the platelet transfusion refractoriness was resolved too.

Conclusion. DSA were detected in 1/8 of USCT candidates (12%) and 2/11 of HaploSCT candidates (18%) and they were associated with failure to obtain allogeneic engrafment in all the 3 cases. An history of platelets transfusion refractoriness was a hallmark of DSA positivity in 2 out of 3 cases. Desensitization treatment achieved DSA cleareance and engrafment of second reinfusion from the same haploidentical donor in 1 patient.

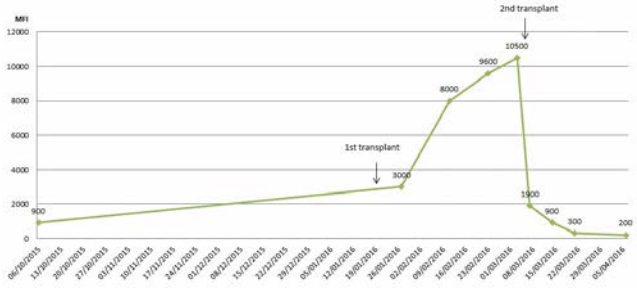

Figure 1.

P103

INCIDENCE AND OUTCOME OF BACTERIAL BLOOD STREAM INFECTIONS IN ALLOTRANSPLANTED PATIENTS: A RETROSPECTIVE, SINGLE CENTER EXPERIENCE FROM 2010 TO 2015

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Introduction: Blood stream infections (BSIs) represent a common event during allogeneic stem cell transplantation (allo-SCT) and monitoring of the single transplant Centre bacterial epidemiology is crucial for patients management.

Methods. We retrospectively analyzed positive blood cultures (BCs) from patients allotransplanted from January 2010 to December 2015 in our Institution. Antimicrobial prophylaxis was conducted according to the published guidelines. GVHD prophylaxis consisted on cyclosporine and metotherxate in all the cases, with the addition of ATG in patients transplanted from matched unrelated donor (MUD). At fever onset, BCs were collected from peripheral blood (PB-BCs) and central venous catheter (CVC-BCs). CVC related infections were defined by a positivity at CVC-BCs 2 hours before the PB-BCs collected at the same time.

Results. 162 patients underwent allo-SCT, of whom 83 (51%) had a positive BCs, for a total of 119 samples. Fifty-two out of 83 patients (63%) had AL or MDS; 30 (36%) and 36 (43%) out of 83 patients were transplanted in first CR and in advanced phase, respectively; 34 (41%) and 49 (59%) out of 83 patients received a myeloablative and reduced-intensity conditioning, respectively. The stem cell source was BM in 17 (20%), PBSC in 60 (72%) and CB in 6 (7%) cases. Donors were sibling in 27 (33%), MUD in 44 (53%) and haploidentical in 6 (7%) cases. The median follow up was 16 months (range 0-68) and the median time to positive BCs since allo-SCT was 19 days (range -4 - +921). In 44/83 patients (53%) we observed a correlation between the BCs and an organ involvement (mainly the lung in 30% of the cases). Seventy-seven (65%) and 42 (35%) out of 119 BCs were sustained by a Gram positive and a Gram negative agent, respectively. The distribution and the antimicrobial sensitivity of the different species is reported in Table 1. Briefly, S. epidermidis and E. coli were the most frequently isolated Gram positive and Gram negative bacteria, respectively (35% and 20%). 67% of E. coli were ESBL positive and 92% were resistant to fluoroquinolones. Moreover 40% and 90% of P. aeruginosa were resistant to carbapenems and to fluoroquinolones, respectively. Sixty (50%) and 59 (50%) out of 119 BCs were CVC-BCs and PB-BCs, respectively. Regarding positive CVC-BCs, 28 (24%) were CVC related BSIs; CVC contamination was observed in 21 cases (18%). The most common isolate from CVC-BCs was S. epidermidis, with 25 cases (21%). Overall, the infection-related mortality was 18% (15 cases). Species with the higher mortality rate were: P. aeruginosa (50%) and E. faecium (67%).

Conclusion. In our cohort of patients we observed a predominance of Gram positive BSIs (namely S. epidermidis), the majority of which were CVC related. The problem of resistance was considerable, in particular against fluoroquinolones and for penicillins. Prospective surveillance of BSIs should be considered a mainstay in the management of patients addressed to allo-SCT.

Table 1. Bacterial isolated and antimicrobial sensitivity.

	Number of BCs	% BCs	% Methicillin-resistance	% Penicillin*-resistance	% Quinolones-resistance
GRAM positive					
TOT	77	65	//	//	//
Staphylococcus epidermidis	42	35	100	100	90
Staphylococcus haemolyticus	9	8	100	100	100
Staphylococcus hominis ssp hominis	8	7	75	75	100
Enterococcus faecalis	6	5	//	33	100
Enterococcus faecium	3	2	//	33	100
Corynebacterium jeikeium	3	2	//	100	100
Corynebacterium striatum	1	1	//	0	0
Corynebacterium urealyticum	1	1	//	0	0
Bacillus firmus	1	1	//	0	0
Staphylococcus aureus	1	1	100	0	100
Staphylococcus warneri	1	1	0	0	0
Difteroidi	1	1	//	0	0
			% ESBL pos	% Carbapenems-resistance	% Quinolones-resistance
GRAM negative					
TOT	42	35	//	//	//
Escherichia coli	24	20	67	0	92
Pseudomonas aeruginosa	10	7	//	40	90
Acinetobacter baumanii	1	1	//	0	100
Enterobacter cloacae ssp cloacae	1	1	//	0	0
Rhizobium radiobacter	1	1	//	0	0
Klebsiella pneumoniae	1	1	0	0	0
Serratia liquefaciens	1	1	//	0	0
Proteus mirabilis	1	1	//	0	0
Salmonella ssp	1	1	//	0	0
Stenotrophomonas maltophila	1	1	//	0	0

* Penicillin: in particular aminopenicillin and ureidopenicillin.

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EARLY FULL DONOR CHIMERISM AFTER ALLOGENEIC STEM CELL TRANSPLANTATION PREDICTS PROLONGED PROGRESSION FREE AND OVERALL SURVIVAL IN ACUTE MYELOID LEUKEMIA

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Introduction: In Acute Myeloid Leukemia (AML) the chimerism status after Allogeneic Hematopoietic Stem Cell Transplantation (HSCT), is currently investigated on myeloid compartment, as whole bone marrow blood sample. Moreover, the prognostic impact of CD3 + T cell chimerism is well studied in Reduced Intensity Conditioning HSCT, but not in myeloablative regimens. Therefore we studied the long-term impact and the kinetics of early chimerism on bone marrow (BM) and CD3+ T cell fraction after AML HSCT.

Methods: We studied 189 patients (167 AML and 22 MDS), transplanted at Hematological Division of Udine between December 2006 and December 2015. The majority received a myeloablative conditioning regimen (81%) and donor was HLA-identical sibling

for 57% patients, unrelated matched for 35% and related haploidentical for 8%. We analysed hematopoietic chimerism at 30, 60, 90 days after transplantation and we included in the study only patients with almost one available chimerism evaluation. We obtained the CD3+ T cell fraction from peripheral blood Ficoll-hystopaque separated lymphocytes by immunomagnetic isolation method (MiltenyiBiotec) and we verified the purity by flow citometry. We performed chimerism analyses by amplification of STRs sequences using AmpflSTRIdentifiler kit (Life Tchnologies) or Power Plex 16HS System (Promega). Full donor chimerism (FDC) was defined as the presence of more of 95% donor cells.

Results: At first month, we evaluated BM chimerism in 144 patients (76%) and CD3+ T cell chimerism in 82 patients (43%). At this time point, 85% of BM and 61% of CD3+ T cells were FDC with a mean value of 97.3% donor (±7.3) in BM and 89.1% donor (±18.9) in CD3+ T cells. We observed that CD3+ T cells chimerism levels at first month ($p<0.00001$) and at second month ($p=0.005$) were significantly lower than BM chimerism level and these differences disappearead at 3 months. Eighty-five patients died at median time of 18 months (range 1-118). The Overall Survival (OS) curves didn't show any significant difference between mixed and FDC status at first month in CD3 or BM. Sixty-one patients (32%) relapsed after HSCT, at a median time of 10 months after HSCT (range 0-93 months). Patients who had FDC CD3+T cells at day 30 had a significantly better Progression Free Survival (PFS) ($p=0.004$). OS and PFS based on BM chimerism status at day 30 was not significant, but BM FDC at 60 and 90 days showed a prolonged 5-year OS and PSF ($p<0.0001$, $p<0.0001$).

Conclusions: We conclude that in a case series of AML patients receiving mainly myeloablative conditioning, CD3+ T cells donor chimerism at day 30 was significantly lower than BM. FDC on CD3+ T cells was the earliest predictor for outcome, since FDC on CD3+ T cells at day 30 predict a prolonged 5 year-PFS. Moreover, FDC on BM at 60 and 90 days was associated with an improved OS and PFS, probably due to stabilization of donor hematopoiesis against leukemia relapse.

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MANAGING NEUTROPENIA BY PEGFILGRASTIM IN PATIENTS AFFECTED BY RELAPSED/REFRACTORY MULTIPLE MYELOMA TREATED WITH BENDAMUSTINE-BORTEZOMIB-DEXAMETHASONE

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The objective of this study was to evaluate the efficacy and safety of pegfilgrastim in relapsed and refractory MM (rrMM) patients, in treatment with courses of Bendamustine-Bortezomib-Dexamethasone (BVD), in order to determine whether pegfilgrastim is as effective as filgrastim, in terms of haematological toxicity, febrile neutropenic episodes, antibiotic usage, and hospitalization duration. From December 2012 to February 2016, 47 patients have been considered (25 M/22 F) with a median age of 61.3 years (r.37-83) affected by rrMM, treated with several lines of treatments (median 6, r. 2-11), and refractory to the drugs previously received, who were treated with monthly courses of BVD (Bendamustine 90 mg/sqm i.v. days 1 and 2, Bortezomib 1 mg/sqm s.c. days 1, 4, 8, 11 and Dexamethasone 20 mg per os days 1, 2, 4, 5, 8, 9, 11, 12, until progression). 24 consecutive patients received pegfilgrastim (6 mg) subcutaneously with a single administration on day + 4, and they were compared to a historical group of 23 consectuve patients in which filgrastim (5 µg/kg/day for at least 3 days) had been given "on demand" if neutrophils count was < $1000x10^9$ cells/L. All patients performed blood counts twice weekly and received, from day +8 to day +19, prophylactic oral quinolones and anti-fungal drugs. In filgrastim group, nadir neutropenia was registered after a median of 9.1 days (r.8-15), with maximum duration of 13 days (median 9.4, r. 7-13); median of nadir neutrophil count was $1.15x10^9$ cells/L (range 0.3-$1.5x10^9$ cells/L). Median number of filgrastim administrations was 4.2 (r.3-6). Patients have been evaluated after at least 3 courses of therapy (r. 3-6). 3 hospitalizations for pneumonia were needed during filgrastim. 4 patients (16.6%) were not able to perform the scheduled chemotherapy treatment because of neutropenia. In pegfilgrastim group, nadir neutropenia, registered at day + 11, was $1.484x10^9$ cells/L (range 1.04-$2.33x10^9$ cells/L). During pegfilgrastim, neutropenia, when present, was shorter than during filgrastim treatment, never longer than 8 days (median 5.9, r. 4-8), with a consequent reduction of neutropenia-related infections. only 4 patients (16.6%) needed, after pegfilgrastim, a supplement of 3 administrations of filgrastim. Patients have been evaluated after at least 3 courses of therapy (r. 3-6). Apart from the advantage of mono-administration, pegfilgrastim was well tolerated in all patients : main side effects were mild fever and bone pain (3/24 : 12.5%), treated with paracetamol. Moreover, no hospitalization was needed during pegfilgrastim. Only 2 patients (8.3%) were not able to comply with the scheduled chemotherapy treatment because of neutropenia. Primary prophylaxis with pegfilgrastim, in patients affected by rrMM, treated with BVD, seems to reduce the incidence and length of neutropenia and the hospitalization. Moreover, it is better tolerated and may increase the opportunity to maintain the programmed treatment.

P106

IMMUNE RECONSTITUTION AFTER ALLOGENEIC STEM CELL TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA: ASSOCIATION WITH TRANPLANT-RELATED COMPLICATIONS, MINIMAL RESIDUAL DISEASE AND SURVIVAL

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Introduction. Post tranplant dysregulation of the immune system limits the success of hematopoietic stem cell transplantation (HSCT) by increasing the risk for GvHD, infections and relapse. Immune cell subsets, including T-, B-, NK- and dendritic cells, have been implicated in the pathogenesis of GvHD, infections and clearance of tumor cells in animal models. The same statement is a challenge in humans. Indeed, studies correlating immune cell subset counts in HSCT- recipients and transplant -related complications are needed. We prospectively investigated post transplant changes in lymphocyte subsets, hematogones and dendritic cells in bone marrow (BM) samples of patients with acute myeloid leukemia (AML) and their associations with minimal residual disease (MRD), GvHD, infections and HSCT type. Then we evaluated which of the immune cell subset in addiction to clinical factors, may influence the survival.

Methods. BM samples from patients (n=25) with AML who underwent allo-HSCT were collected from january 2012 to december 2014 at specific time-points: +1 , +3 and +6 months from transplant. B cells, hematogones (HGs), helper T cells (Th), cytotoxic T cells (Tc), regulatory T cells (Tregs), natural killer (NK) cells, dendritic cells (DC, plasmocytoid (DPC) and myeloid (DMC)) as well as MRD were investigated using 6-color flow-cytometry. Results. NKs displayed values higher than 2.21%, 2.56% and 2.45% at +1, +3 and +6 mths from transplant, respectively. Th were less than 4.6% at each time point while Tc abnormally increased only after 3 months from HSCT. Levels of Tregs raised over 9.72% at + 1 mth and reduced later. HGs increased over 1.55% and 1.84% at +3 and +6 mths respectively. No significant changes were observed for B lymphocytes. Only DPC showed counts lower than 0.20% at +3 and +6 mths. Patients with aGvHD showed a significantly higher probability (100%) to have NK cells over 2.21% at +1 mth com-

pared to the others (52.9%)(p=0.05). Patients who underwent sibling HSCT had a significantly higher probability (90.9%) to have HGs greater than 1.55% in comparison to not siblings (36.4%)(p=0.02). Conversely, HSCT -recipients with cGvHD showed HGs significantly lower than 1.55% (28.6% vs 80%, p=0.05). MRD, HGs and relapse demonstrated to be predictive factors of prognosis. Patients having a MRD greater than 0.05% at +1 mth showed a significantly shorter DFS(p=0.02) and OS (p=0.02). HGs increased over 1.55% at +3 mths predicted a better survival while the occurrence of relapse defined the highest risk of death (p<0.01).

Conclusion. Our results suggest a role of NKs in the pathogenesis of aGvHD. HSCT type and cGvHD differently influenced the number of hematogones which represented a very useful indicator of subsequent survival. Positive MRD at +1 mth strongly predicted a worst outcome of HSCT-recipients. Unfortunately, post-transplant immunity did not seem to act on MRD. A strategic approach to prevent the relapse should be mandatory.

P107

LENALIDOMIDE THERAPY IN MYELODYSPLASTIC SYNDROMES WITH LOW-OR-INTERMEDIATE-1 IPSS RISK AND DEL(5Q): RETROSPECTIVE STUDY OF 17 PATIENTS FROM A SINGLE INSTITUTION

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Introduction. Lenalidomide is approved for the treatment of symptomatic anemia in patients (pts) with lower-risk myelodysplastic syndromes (MDS) with del(5q), but more data from real-life and registry studies are needed. Therefore, we retrospectively analyzed the clinical and laboratory data of MDS pts with del(5q) treated with lenalidomide in our Center. Methods. The type of response was defined according to IWG criteria (Cheson 2006): Complete Remission (CR), Partial Remission (PR), Marrow CR (mCR), Hematologic Improvement (HI). Moreover, we quantified the degree of phosphoinositide-phospholipase C (PI-PLC) beta1 methylation and gene expression before and during lenalidomide administration. Results. From July 2009 in our Institution 17 MDS pts (5 males, median age 80, range 69-90 yrs) with del(5q), either isolated (11 pts) or associated with another single additional cytogenetic abnormality (6 pts) were treated with lenalidomide. At the start of treatment, IPSS risk was: low in 8 pts, and intermediate-1 in 9 pts; IPSS-R risk was : low in 10 pts and intermediate in 7 pts. MDS-specific comorbidity index (MDS-CI) risk score was low in 8 pts, intermediate in 7 pts, and high in 2 pts. The median interval between diagnosis and the start of treatment was of 5 (2-153) months. 16/17 pts had received RBC transfusions before the start of treatment; pre-treatment median number of RBC transfusion was 8.5 (1-616), over a median time of 20 (2-616) weeks. The pts received a median of 5 (1-56) cycles of lenalidomide, with a median duration of treatment of 8 (1-63) months. 10/17 pts (58.8%) completed at least 4 cycles of therapy and were considered evaluable for response. 2 pts are still on therapy, but have not yet completed 4 cycles of treatment. 5 pts (29.4%) discontinued lenalidomide before the 4th cycle because of stroke (1 pt), brain tumor (1 pt) cutaneous rash (1 pt), thrombocytopenia (1 pt), gastrointestinal intolerance (1 pt), respectively. All the 10 evaluable pts showed a favourable response (ORR: 100%). The best hematological response achieved was CR in 6 pts and HI in 4 pts. The median maximum Hb increase was 3.7 (1.9-7.7) g/dL. A first response was observed after a median of 2 (1-5) cycles. 5 pts showed a complete cytogenetic response and 4 a partial cytogenetic response (Cheson 2006). The median duration of response is 7 (5-78) months (5 pts still in response), with a median follow-up of 14 (1-80) months. A grade > 2 haematological and non-haematological toxicity was observed in 13 (76.5%) and 3 (17.6%) pts, respectively. 1 pt (5.9%) showed progression to AML after 12 months. Responder pts showed a molecular activation of erythropoiesis, in that Phospholipase C gamma1, Beta-Globin and Glycophorin A levels increased, and also displayed a specific phosphorylation of Akt only in non-del(5q) cells. Conclusions. Our results confirm the efficacy and safety of lenalidomide even in a unselected elderly population of lower-risk MDS pts with del(5q).

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PU.1 EXPRESSION CORRELATES WITH PATIENT DISEASE STATUS IN THE MYELODYSPLASTIC SYNDROMES, A NEW PROGNOSTIC MARKER?

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Background: Myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem cell disorders characterised by ineffective haematopoiesis and dysplasia, manifesting as variable degrees and combinations of peripheral blood cytopenia. The result is often transfusion-dependent anaemia, increased risk of infection, bleeding complications, and an increased potential of progression to acute myelogenous leukaemia (AML). In recent years treatment with 5-azacytidine (AZA) has seen an increase in patient survival for intermediate and high-risk group MDS patients, although the precise mechanism of action is not yet fully understood. Previous studies have demonstrated down regulation of the PU.1 transcription factor in high-risk MDS patients, which can be reversed by administration of AZA. However, it is not currently known if PU.1 levels correlate with MDS disease severity and/or prognosis.

Aims: Assess if PU.1 expression levels in MDS patients correlate with disease prognosis as determined by risk group stratification using the Revised International Prognostic Scoring System (IPSS-R). In addition, we will use commercially available cell lines (SKM-1, MOLM-13, K562, HL60) to explore the potential of AZA in correcting down-regulated PU.1 expression that is seen in high-risk MDS.

Methods: BM specimens were collected from 13 patients diagnosed with MDS who were stratified according to IPSS-R guidelines (5-low, 3-int, 2-high risk) and from 13 haematological normal controls. Samples were enriched for the mononuclear fraction by Ficoll separation. Total RNA was extracted and analysed by Real Time PCR for PU.1 expression relative to the housekeeping gene GAPDH using the 2-ΔΔCT method. In vitro models of MDS, SKM-1 and MOLM-13 were treated with 1 μM AZA for 24, 48, or 72hr followed by analysis of PU.1 expression analysis by RT-qPCR.

Results: Analysis of patient samples revealed that PU.1 expression is significantly lower in high-risk patients compared controls. In addition, preliminary data suggests that PU.1 expression also correlates with disease severity and could provide insight as a prognostic marker. PU.1 expression was significantly increased upon treatment with 1 μM AZA in commercially available cell lines.

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ROLE OF THE IL28B RS12979860 C/T POLYMORPHISM ON THE CYTOMEGALOVIRUS REACTIVATION IN AUTOLOGOUS STEM CELL TRANSPLANT PATIENTS

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Background: CMV infection represents one of the main cause of morbility and mortality after stem cell transplantation. Type III interferons (IFNs), including IFNℓ1 (IL29), IFNℓ2 (IL28A) and IFNlℓ3 (IL28B), are thought to display potent antiviral and immunemodulatory properties *in vivo*, which may overlap partially with those exerted by type I IFNs. Type I and Type III IFNsℓ both generate an antiviral state by triggering the JAK-STAT pathway, ultimately upregulating the expression of interferon-stimulated genes. Rs12979860 single nucleotide polymorphism (SNP) in IL28B gene region is well known to influence the spontaneous and treatment-induced clearance in HCV infection. Data on the relevance of such a SNP in other viral infections is still debated even, Bravo et al. recently documented a protective effect of the T allele against CMV infection in the Allogenic stem cell transplantation (Allo-SCT) (Journal of Medical Virology 2014.86:838).

Aim of the study: The current study was aimed at investigating whether the IL28B polymorphism Rs12979860 may effect on the CMV reactivaction in the Autologous stem cell transplantation setting.Patients and methods: From October 2014 67 patients were included in the study because underwent a autologous stem cell transplantation for hematological diseases. The median age of the patients was 56 years (16-66 years). Patients were distributed according to Hematologic disease as follows: 75% of the patients had Multiple Myeloma, 16% non Hodgkin Lymphoma, 6% Hodgkin Lymphoma e 3% Acute Myeloid Leukemia. The Rs12979860 IL28B SNP (C/T) genotype was determined by Melthing analysis on DNA derived from peripheral blood samples. CMV DNAemia was determined by quantitative Real-Time PCR with a limit detection of 50 copies/mL (Artus, Qiagen). Patients were monitored for CMV DNAemia weekly for the three months after stem cell transplantation.

Results: CC genotype was detected in 46% of patients, CT genotype in 40% and TT genotype only in 14 % of patients according to the lowest frequency of TT genotype harboring in general population. A CMV reactivation (syntomatic and asyntomatic) was documented respectively in 77.7%, 67.7% and 44.4% of patients carrying TT, CC and CT genotype. A trend towards a higher incidence of CMV reactivaction was noted in the TT population with respect to CT and CC population (P=NS). Comparing the patients with the carriage of Il-28B CT versus CC plus TT genotype, there is an higher incidence of CMV reactivation in those patients with CC plus TT genotype according to logistic regression (44.4% versus 70% ; P .039,CI 95%, OR 2.91) (codominat genetic model).

Conclusions. In conclusion, our data suggest a protective effect of the carriage of IL-28B C/T genotype against CMV infection in the ASCT setting.

P110

COMPARATIVE STUDY ON ATG THYMOGLOBULIN VERSUS ATG FRESENIUS FOR THE GRAFT VERSUS HOST DISEASE (GVHD) PROPHYLAXIS IN ALLOGENEIC STEM CELL TRANSPLANTATION FROM MATCHED UNRELATED DONOR: A SINGLE CENTRE EXPERIENCE

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Introduction. Graft-versus-host disease (GVHD) is one of major causes of morbidity and mortality after allogeneic stem cell transplantation (allo-SCT). ATG-Thymoglobulin (ATG-T) and ATG-Fresenius (ATG-F) are two rabbit anti-thymocyte globulins (ATGs) that are currently used in the conditioning regimen of allo-SCT for the prevention of GVHD. There are no randomized studies comparing the efficacy and safety of ATG-T and ATG-F in patients undergoing allo-SCT from matched unrelated donor (MUD) and there is only one comparative study about it. In our retrospective study we analyzed the incidence of acute and chronic GVHD, the side effects and the outcome in two cohorts of patients who received ATG-T or ATG-F.

Methods. Between March 2009 and December 2014 a total of 77 patients underwent allo-SCT from MUD and received pre-transplant ATG-T (n = 31) at a total dose of 10 mg/Kg or ATG-F (n = 46) at a total dose of 30 mg/Kg. The two groups of patients were comparable regarding age, disease, HLA-match, stem cell source, disease distribution and disease status at time of transplant.

Results. No significant difference was observed in the incidence of acute GVHD (aGVHD) (p=0,81) and grade III-IV aGVHD (p=0,39). Eight (31%) out of 26 patients treated with ATG-T and ten (46%) out of 39 patients treated with ATG-F who survived over the 100-day post-transplant, developed chronic GVHD (cGVHD). A moderate-severe cGVHD occurred in 6/8 (75%) patients treated with ATG-T and in 3/10 (30%) patients treated with ATG-F. No significant differences were observed in the incidence of bacterial, viral and fungal infections, relapse rate and overall survival. The most frequent side effects such as fever, chills, diarrhea with abdominal pain or skin rash were recorded in a greater proportion of ATG-F patients (32% vs 67%, P<0,01). Anyway side effects were mild, controlled by steroid therapy and none led to discontinuation of ATG.

Conclusions. Our data suggest that clinical outcome was quite similar for patients who received ATG-T or ATG-F prophylaxis. However the ATG-F group showed a trend towards a lower incidence of moderate-severe cGVHD. This latter observation confirm the results recently published in the other only one study comparing ATG-T and ATG-F.

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IRON CHELATION THERAPY IMPROVES HAEMATOLOGICAL RESPONSE IN HIGH-RISK MYELODYSPLASTIC PATIENTS TREATED WITH AZACITIDINE

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Background. The goals of treating older patients with myelodysplastic syndrome (MDS) are different than for younger patients. Few elderly patients are able to pursue an allogeneic stem cell transplant. Azacitidine (AZA) improves long-term outcomes of higher-risk MDS patients and is now the reference frontline therapy of higher-risk MDS not eligible for allogeneic stem cell transplant. Anaemia is the most common symptom of MDS and most

patients become transfusion-dependent with the risk of iron overload. Deferasirox is an orally available iron chelator administered once-daily in transfusion-dependent patients with various chronic anaemias. Its efficacy has been established in controlled clinical trials.

Aim. We report our experience on using the azacitidine in patients with high-risk MDS, evaluating the efficacy and safety. Concomitant treatment with deferasirox was performed in a routine clinical setting following Consensus Guidelines on Iron Chelation Therapy.

Methods. In our Institution from October 2009 to April 2016 we have treated 30 elderly patients (19 male and 11 female, median age 76 years, r. 72-88) affected by HIGH-RISK MDS (IPSS INT-2/HIGH). Patients received subcutaneous azacitidine at 75mg/m(2) daily for 7 days every 4 weeks. All patients completed at least 6 cycles of therapy. 12/30 (40%) patients underwent more than 8 cycles of therapy. 18/30 patients underwent as well iron chelation therapy with deferasirox receiving a starting dosage of 10 mg/kg/day, subsequently titrated according to serum ferritin (SF) measured monthly.

Results. Complete response (CR), partial response (PR), and hematologic improvement (HI) were observed in 2 (7%), 5 (17%), and 12 (40%) patients, respectively. The median number of cycles to clinical response was 4 (range 4-8). The 2-year rate of acute myeloid leukemia-free survival was 48%. Five serious adverse events occurred in five patients with one fatal outcome. 16 out of 18 patients who showed any hematologic response (CR+PR+HI) meeting International Working Group 2006 criteria had also performed deferasirox therapy. No increased toxicity was noted when deferasirox was used concomitantly with azacitidine.

Conclusions. Our results confirm the effectiveness of the therapy with azacitidine in HIGH-RISK MDS elderly patients with acceptable toxicity profile. Peripheral cytopenias were the most commonly occurring adverse event, with gastrointestinal adverse events and injection-site reactions among the most commonly occurring non-haematological adverse events. In conclusion, azacitidine is an important agent for use in the treatment of elderly patients with MDS. Furthermore concurrent use of deferasirox in patients with iron overload seems to significantly improve the hematologic response by reducing transfusion requirement.

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INFLUENCE OF UNMANIPULATED HAPLOIDENTICAL, UNRELATED AND MATCHED SIBLING GRAFTS ON OUTCOME IN ACUTE MYELOID LEUKEMIA. A SINGLE CENTER EXPERIENCE

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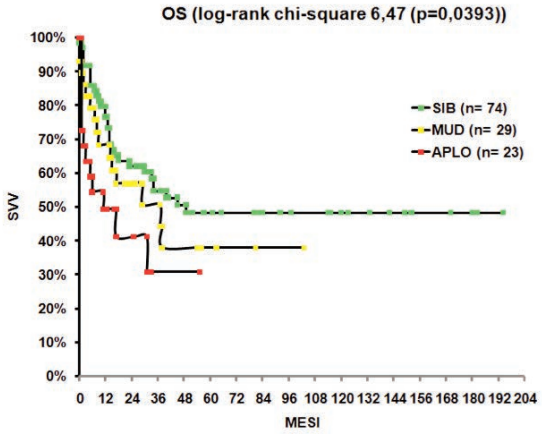
Introduction: Allogeneic stem cell transplant (allo-SCT) provides a potentially curative therapy for patients with high-risk or chemorefractory acute myeloid leukemia (AML). In the last years several transplant strategies, using alternative donors or different conditioning regimens, have been introduced into clinical practice to improve outcome in haematological malignancies, especially in AML. The aim of the present study was to evaluate the impact of HLA identical siblings (SIB) unrelated donors (UD)(8/8 HLA-matched) and HLA haploidentical family donors (HAPLO) on survival, transplant related mortality (TRM) and quality of response in patients affected by acute myeloid leukemia (AML).

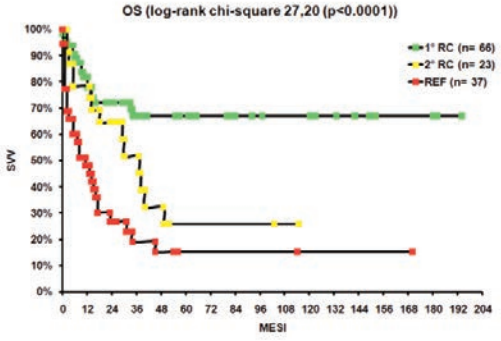
Material (or patients) and methods: The study population was represented of 126 AML patients receiving allogeneic transplant (SCT) in a single Center. Of them, HAPLO were 22, SIB were 74 and UD were 30. Patients were stratified on the basis of disease phase: first remission (CR1, n = 66), second remission (CR2, n = 23) or refractory disease (Ref, n = 37). The conditioning regimen was defined as myeloablative (MAC) (n = 113) or reduced intensity (RIC) (n = 13). In order to prevent Graft vs host disease (GvHD), the following prophylaxis were administered: unmanipulated bone marrow, post transplant cyclophosphamide (PT-CY) with cyclosporine (CsA) and mycophenolate (MMF) in HAPLO, CsA and methotrexate (MTX) in SIB and CsA +MTX+antithymocyte globulin (ATG) in UD. Fifteen (68%) HAPLO recipients were refractory and six (27%) of them received a previous transplant (a (autologous n = 5; allogeneic SIB n = 1).

Results: The 3 -years OS on the entire study- cohort (n = 126) was 51,5%, although significant differences were noticed among the three donor types of transplant: 3 -years OS was 60%, 51% and 30,5% (P = 0,0393) for SIB, UD and HAPLO graft, respectively. When the response quality was considered, CR1 showed a longer OS compared to CR2 and refractory patients (73% vs 51% vs 19%, respectively (p>0.0001).The cumulative incidence (CI) of TRM was 19% in all patients but TRM was lower in SIBs (12,1%) respect to HAPLO (27,2%) and UD (30%). Similar results came from the comparison between disease phase and outcome: 13,6%, 26% and 24,3% for CR1, CR2 and refractory disease, respectively.

Conclusion: In conclusion, the early phase of response was a positive predictor of a better OS compared to refractory disease. SIB showed the best outcome while no significant differences were found between HAPLO and UD transplantation.

Disclosure of Interest: None declared.





ANALISI MULTIVARIATA OS

Covariate	p
RC	0,00009
sib 1 mud 2 aplo 3	0,137284517

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ZINC SUPPLEMENTATION PREVENTS VIRUS REACTIVATION (TTV) AND CD8+ SLUMP AT DAY 100 AFTER AUTO-SCT: FIRST RESULTS FROM ZENITH (ZINC AS ENHANCER OF IMMUNE RECONSTITUTION AFTER TRANSPLANTATION FOR HAEMATOLOGICAL MALIGNANCIES) STUDY

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Introduction: Immune reconstitution after stem cell transplantation (SCT) plays a crucial role in host defense against microbial agents. Many efforts have been made to identify the role of specific subsets of T cells responsible for anti-microbial, anti-tumor and graft-versus-host effect. However, the thymus atrophy in adult represents a limit for T cell reconstitution from precursors. The reactivation of thymic function after BMT is documented but the proper strategy to improve the thymic output is currently matter of debate. Pre-clinical and clinical evidences suggest that Zinc may contribute to thymic reactivation and to improve the T-mediated cellular defense against pathogens. In this study, we tested the role of Zinc oral supplementation in immune reconstitution after autologous SCT in multiple myeloma.

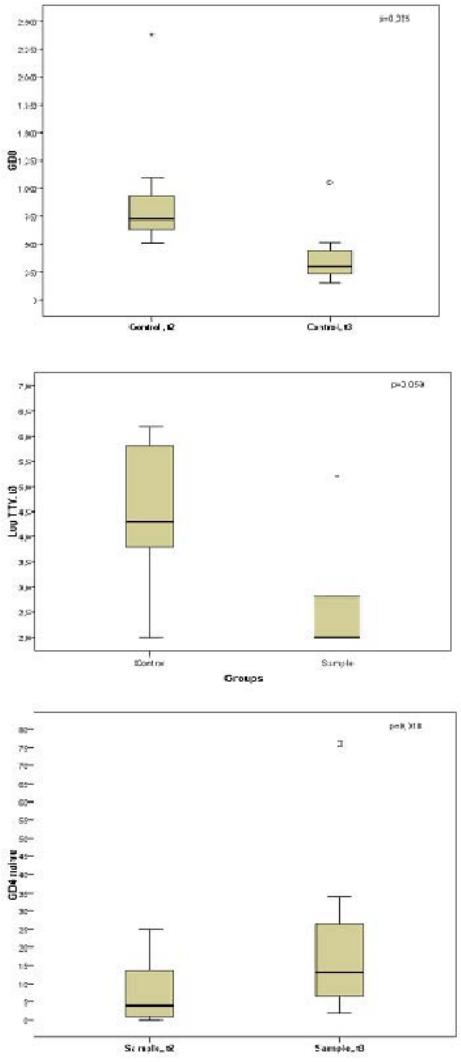

Figure 1.

Methods: From December 2013, we prospectively enrolled 18 patients (12 male, 6 female; average age: 58 years, range 43-72) undergoing single MEL 200 auto-SCT after one or two lines of therapy. The protocol was approved by local ethics committee (EUDRACT: 2014-004499-47). All patients undersigned an informed consent. Randomization was effected in day 0. Nine patients were treated from day 5 to day 100 with 600 mg/day of zinc sulfate (uncoated tablets), whereas nine patients received only standard antimicrobial prophylaxis. The average age was similar in the two groups (58 years in control group, 63 years in the sample). Peripheal blood samples were collected in both groups at day 30 (t2) and day 100 (t3). Eight-colour flow cytometry was performed for CD3, CD4, CD8, CD45RA, CD45R0, CD27, CD28. The lymphocyte populations absolute count was statistically analyzed intra- and inter-group. A qPCR for viral load of Torquetenovirus (TTV), a harmless virus whose viral load is related to immunodepression, was performed. Zinc serum level was measured.

Results: the recovery of naïve CD4 cells is visible in both groups from day 30 to day 100 but a significant increase is detected only in the sample group. CD8+ T cells shown a notable decrease until day 100 in the control group, specifically in CD8 naïve, memory, effector memory populations. TTV load increases in inverse proportion to the decrease of CD8 population. In the Zinc group there are no differences between the TTV load at the two timepoints. As a result, the viral load of TTV is higher in the control group than in the Zinc group at day 100. Zinc serum levels were normal in all patients.

Conclusions: data show that zinc may play a role in a faster recovery of CD4 naïve T cells. Furthermore, zinc seems to prevent the expected CD8 decrease of the control group. The TTV reactivation could be explained by the impaired CD8 cytotoxic activity, on the base of the inverse correlation between viral load and CD8 lymphocyte count. Zinc-deficiency was not observed in any of the patient, however a Zinc supplementation could contribute to a better reconstitution. To the best of our knowledge, this is the first study describing the role of Zinc in stem cell transplantation. More molecular and functional data are needed to demonstrate a possible role of Zinc in the transplant setting and in thymic lymphopoiesis.