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PROCEEDINGS

THE ITALIAN SOCIETY OF CYTOMETRY
GIC

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R. DE VITA and G. MAZZINI

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FLOW CYTOMETRY IN THE STUDY OF TUMOR-HOST INTERACTIONS: THE EXAMPLE OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Chronic lymphocytic leukemia (CLL) is one of the most outstanding example of neoplasia in which the genetic asset of tumor cells and the host microenvironment almost equally contribute to disease progression. Indeed, leukemic cells need to be surrounded and supported by non-tumoral cells to survive and proliferate and these "dangerous liaisons" take place mainly in privileged niches within the lymph nodes. Here multiple elements, including stromal cells, monocyte-derived nurse-like cells and T-lymphocytes, interact with CLL, and leukemic cells play a central role in shaping a tumor-friendly immune-tolerant environment. In particular, CLL is associated with marked T cell dysfunctions, with abnormalities in the phenotype of $CD4^+$ and $CD8^+$ populations and defects in the generation of competent cytotoxic T cells. Leukemic patients show a disequilibrium in the T cell subsets compared to healthy subjects, with the accumulation of terminally differentiated effector memory T cells and a decrease of naïve precursors. Furthermore, as evaluated by a multiparametric flow cytometry approach, $CD4^+$ and $CD8^+$ T cells in CLL express high levels of the programmed death-1 (PD-1) receptor and high PD-L1 expression is found on the B-cell counterpart, and this crosstalk significantly impairs IFNγ production in $CD8^+$ T lymphocytes.
Among others, the adenosinergic axis significantly contributes to immune suppression by acting on the T cell compartment. Specifically, the adenosine produced by CD39 and CD73 expressed on the surface of $CD4^+/CD25^+/FoxP3^+$ Treg cells, suppress the activity of effector T cells. Adenosine is also produced by CLL cells themselves and results in a autocrine/paracrine signaling through binding of specific adenosine receptors expressed on leukemic cells, T cells and nurse-like cells. Beside the direct effects on CLL cells, adenosine promotes skewing of macrophages towards a M2 phenotype and decreases T cell responses.
An important tool to mimic tumor-host interactions is represented by in vivo mouse models, based on the adoptive transfer of leukemic cells into immunocompetent mice. In these models, a flow cytometry-based approach allows to dynamically study the reciprocal modulation occurring between leukemia and bystander cells. Murine leukemic cells can be identified and monitored as the $CD5^+/B220^+$ double positive population in the peripheral blood (PB) that progressively expands. Changes in the T and myeloid subsets can be evaluated by a multiparametric analysis using specific markers to characterize the different subpopulations and their polarization consequently to leukemic engraftment.

EXTRACELLULAR VESICLES (EVS) IN HEMATOLOGIC MALIGNANCIES AND IN HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Extracellular vesicles (EVs) are cell-derived particles released by many different cell types in normal and pathological conditions, including neoplasms. They are vehicles for various types of molecules (proteins, lipids, and nucleic acids) so that they are able to "talk about" presence and nature of origin cells. In some cases, EVs contains a peculiar molecular cargo, thanks to a selective mechanism of cellular packaging. EVs are players in intercellular communications in short and long distance interplays. In hematological malignancies (HMs) EVs can modify and bone marrow microenvironment, improving its effectiveness in supporting malignancy, through immune suppression and multidrug resistance induction. EVs are present in biologic fluids and are able to preserve their molecular content from degradation.
EVs are potential biomarkers in HM. Their levels are significantly elevated in Waldenstrom Macroglobulinemia (WM), Hodgkin Disease (HD), Multiple Myeloma (MM), Acute Myeloid Leukemia (AML), Myeloproliferative neoplasms and, at a lesser degree, in Chronic Lymphocytic Leukemia (CLL) and Non Hodgkin Lymphomas, as compared to healthy controls. EVs from patients specifically retain tumor-related antigens, such as CD19 in B cell neoplasms, CD38 in MM, CD13 in AML, and CD30 in HD. Both total and antigen-specific count of EVs significantly correlates with clinical features such as Rai stage in CLL, International Prognostic Scoring System in WM, International Staging System in MM, and clinical stage in HD.
Mesenchymal stem cells (MSCs) are a heterogeneous cell population containing progenitors able to repair tissues, support hematopoiesis, and modulate immune and inflammatory responses. Several clinical trials have used MSCs in allogeneic hematopoietic stem cell transplantation to prevent hematopoietic stem cell (HSC) engraftment failure, reduce aplasia post chemotherapy, and control graft versus host disease (GvHD). The efficacy of MSCs is due to their immune suppressive and anti-inflammatory properties. Extracellular vesicles (EVs) from MSCs mediate most of these effects. MSC-EVs may offer specific advantages for patient safety, such as lower propensity to trigger innate and adaptive immune responses. It has been also shown that MSC-EVs can prevent or treat acute-GvHD by modulating the immune-response and, combined with HSCs, may contribute to hematopoietic microenvironment reconstitution.

CHROMOSOME GENOMICS IN THE POST-GENOMIC ERA

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Chromosome genomics exploits the organization of plant genomes into subunits called chromosomes to overcome difficulties due to large genome size and DNA sequence redundancy. Purification of individual chromosomes by

flow cytometric sorting reduces DNA sample complexity and greatly simplifies genome mapping, sequencing and gene isolation. This approach is particularly useful in polyploids, as it avoids problems due to homoeologous sequences. The chromosome-centric approach stimulated rapid advance in genomics of species, which were considered intractable using standard whole-genome strategies, such as bread wheat, barley and rye. The recent progress in DNA sequencing technologies and genome assembly algorithms made the production of reference genomes by shotgun sequencing feasible even in plants with complex genomes. Yet, in species with large genomes, production of whole genome assemblies from multiple individuals remains expensive and chromosome genomics enables targeting particular genome regions. This results in a significant reduction of costs and, if needed, allows analyzing a chromosome of interest isolated from multiple individuals (genotypes, mutants). The applications include identification of chromosomes with integrated transgenes, characterization of alien chromatin in introgression lines and development of molecular markers for marker assisted selection. Gene isolation is becoming one of the most important applications of chromosome genomics. The targeted approach greatly streamlines gene cloning and reduces project costs. Two chromosome-based gene cloning approaches, namely MutChromSeq and TACCA (TArgeted Chromosome-based Cloning via long-range Assembly) have been developed and validated recently. As chromosome genomics can be applied in any species from which a liquid suspension of intact mitotic chromosomes can be prepared, it will continue playing an important role also in the post-genomic era. This work has been supported by the National Program of Sustainability (grant award LO 2014).

T FOLLICULAR HELPER AND T FOLLICULAR REGULATORY CELLS - THE CASE OF MYASTHENIA GRAVIS

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$CD4^+$T follicular helper (TFH) cells assist humoral immune responses by helping B cells to differentiate into plasma cells producing high-affinity antibodies against foreign antigens and mediate the selection and survival of memory B cells. In humans, TFH cells have been originally identified in tonsils and a circulating counterpart (sometimes referred to as TFH-like cells) has been identified on the basis of the cell surface receptor CXCR5 which allows for homing to the B cell zones of secondary lymphoid organs. Studies have reported on a small subset of TFH cells that express FOXP3, the transcription factor controlling regulatory T (Treg) cells. These cells have been named T follicular regulatory (TFR) cells and have originally been reported in the germinal centres of lymph nodes where they counteract the helper function of the TFH cells.
Because the TFH cells are required for germinal centres formation and maintenance, whereas the TFR cells participate in the regulation of germinal centres formation reactions, an imbalanced TFH/TFR ratio may increase the risk of autoimmunity by facilitating autoantibody production. This notion prompted a number of translational studies that used flow cytometry to enumerate and subcategorise these two T follicular cell subsets in a variety of autoimmune diseases characterized by the generation of pathogenic autoantibodies.
In addition to CXCR5, the TFH cells are defined by BCL-6 expression and IL-21 production. However, due to the inherent difficulty in assessing intracellular and functional markers by flow cytometry, as compared to cell surface markers, CXCR5 is the marker of choice in association with various cell surface markers, namely CCR7, PD-1, CXCR3 and CCR6 to identify circulating TFH cells and their subsets in most translational studies. Two major TFH cell subsets are distinguished according to the coordinate expression of CCR7 and PD-1. These subsets are further categorized according to the coordinate expression of CXCR3 and CCR6. Functional significance has been credited to the majority of the subsets.
In peripheral blood, the TFR cells (sometimes referred to as TFR-like cells) can be recognized by the coordinate expression of CD25 and FOXP3, akin the Treg cells. Importantly, TFH cells showing $CD25^{low/high}FOXP3^+$ phenotype largely correspond to TFH cells showing $CD25^{low/high}CD127^{low/neg}$ phenotype, as in the Treg cells. Thus, being FOXP3 an intracellular marker, the $CD25^{low/high}CD127^{low/neg}$ phenotype is commonly preferred for identifying circulating TFR cells in translational studies. Assessing CD45RA expression allows for further categorising TFR cells according to their activation/memory status.
The TFH/TFR ratio has been largely investigated in systemic lupus erythematosus, the prototypical systemic autoimmune disease, as well as in a variety of human pathology, including autoimmune thyroid diseases, rheumatoid arthritis, juvenile dermatomyositis, Sjogren's syndrome, and Myasthenia gravis (MG).
MG is an organ-specific immune disease that is characterized by autoantibodies such as the anti-Acetylcholine receptor antibody (anti-AChR Ab) and anti-Muscle-specific tyrosine kinase antibody (anti-MuSK Ab). Here we will describe changes occurring in the frequency of TFH cells (and their major subsets) and TFR cells (and their major subsets) in patients with MG with a focus on flow cytometry matters such as determining the best placement of TFH/TFR cell markers/fluorochromes in order to prevent fluorochrome spillover, minimize compensation issues while providing bright, discriminatory staining of populations of interest, and the proper use of controls to establish the boundaries of gates to ensure consistent and reproducible data.

CANCER IMMUNOTHERAPY AND FLOW CYTOMETRY: TELOMERASE VACCINES

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Telomerase is the reverse transcriptase responsible for synthesis, elongation and stability of the telomeric regions of chromosomes, normally expressed by embryonic cells but not by adult somatic cells. Importantly, it is re-expressed by cancers since essential for tumor cell immortalization. Telomerase collects interest by tumor immunologists, as it is needed to maintain the unlimited proliferative activity of tumor cells without undergoing apoptosis, and it is expressed by all tumor histologic types: hence, it can represent a universal tumor associated antigen. Hence, it has been proposed as a candidate antigen for anti-cancer vaccination. The studies performed in the last 15 years demonstrated their immunogenicity and prompted clinical trials to evaluate immunological and clinical efficacy of telomerase vaccination.

These trials consistently supported the safety of telomerase vaccines, but the clinical efficacy was not so satisfactory since the rate of responses was low in different studies. However, these studies provided important insights on new possible approaches, related to the formulation of the vaccine, the selection of adjuvant, the clinical setting, to improve the clinical outcome in patients treated by telomerase vaccines. In this view, our group recently completed a phase I/II trial in prostate and renal cancer patients with a newly generated cancer vaccine, named GX301, a multi-peptidic vaccine, including four peptides able to promiscuously bind several HLA class I and II alleles, thus allowing both the coverage of HLA restriction by the majority of haplotypes, and the induction of helper and cytotoxic T cell responses. Moreover, this vaccine exploits complementary activities of two adjuvants, to efficiently activate innate and adoptive immune system. The results of the phase I/II trial performed by several cytometric assays plus ELISPOT, showing a 100% rate of telomerase-specific immune responses in the immunized patients associated with clear evidences of clinical responses in immunologically high responder patients (notwithstanding the very advanced disease and the poor expectancy of life), suggest the need to conduce further studies on telomerase vaccines in order to fully understand the real therapeutic potential of this immunotherapy.

AIEOP-BFM CONSENSUS GUIDELINES 2016 FOR FLOW CYTOMETRIC IMMUNOPHENOTYPING OF PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

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Immunophenotyping by flow cytometry (FCM) is a fundamental tool in Acute leukemia diagnostics. However, a gap exists between different classification systems, method's standardization, and clinical needs. In this context the AIEOP-BFM consortium induced an extensive standardization and validation effort among national reference laboratories collaborating in immunophenotyping of pediatric acute lymphoblastic leukemia (ALL). We elaborated common guidelines for multi-color FCM-based diagnostic immuophenotyping of acute lymphoblastic leukemia (ALL). In particular we assessed several issues such as: marker's panel, immunological blast gating procedures, in-sample controls, antigen expression rating , blast cell heterogeneities and subclone formation. We propose a refined ALL subclassification, and a dominant lineage assignment algorithm able to distinguish "simple" from bilineal/"complex" mixed phenotype acute leukemia (MPAL) cases, which is essential for choice of treatment. These guidelines are a first step towards necessary inter-laboratory standardization of pediatric leukemia immunophenotyping for a concordant multicentric application.

IMMUNOCOMPETENCE ASSESSMENT IN NON-SMALL CELL LUNG CANCER AFFECTED PATIENTS TREATED WITH ANTI-TUMOR IMMUNOTHERAPY

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Background: One of the mechanisms by which tumors can evade immune response is the immune-suppression mediated by the dysregulation of immune-checkpoint proteins expression. In the last years, two immune-checkpoint receptors have been most actively studied, in particular cytotoxic T lymphocyte associated antigen 4 (CTLA4) and programmed cell death protein 1 (PD1). Monoclonal antibodies (mAbs) against CTLA4 and successively against PD-1 have been approved by FDA for Stage IIIB/IV non-small cell lung cancer (NSCLC) patients unresponsive to first line therapies.
Among anti-PD-1 (Nivolumab) treated patients some present a very good and stable response, in others the same treatment results ineffective: based on this observation we hypothesize that by monitoring immunological parameters before, during and after anti-PD1 therapy in NSCLC patients, we could find immunological biomarkers correlating with clinical outcomes.
Material and Methods: We recruited 13 anti-PD1-treated NSCLC patients for longitudinal evaluation (T0, T60, T120, T240 T360 days) of phenotypic and functional features of peripheral blood lymphocytes. In particular intracellular cytokines production and surface molecules expression have been assessed in order to identify the different T lymphocytes subsets and immune-checkpoints, by using the following fluorochrome-conjugated mAbs against CD3, CD8, CD4, CD161, IFN-γ, TNF-α, IL-2, IL-17, IL-4, IL-10, PD1, PD1-L, CTLA-4, CD25, Foxp3, Perforin, Granzime. The ability of T cells to proliferate in response to recall antigens was also evaluated during all the follow-up period.
Results and conclusion: Our preliminary data show that patients treated with Nivolumab tend to enhance their immunocompetence. The patients affected by NSCLC after anti-PD1 treatment showed higher level in frequency of IFN-gamma and TNF-alfa CD8+ and CD4+ producing T cells. Furthermore the frequency of Treg cells was reduced during treatment in responder subjects, similarly also the surface expression of CTLA-4 and of PD-1 on T

cells was downregulated. In conclusion our data show that circulating T cells from NSCLC affected patients, tend to lose a regulatory phenotype, and acquire an effector one during biologic therapy, especially those responsive to Nivolumab.

FLOW CYTOMETRY APPLICATIONS IN FOOD MICROBIOLOGY: NOT ONLY CELL COUNTING!

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The flow cytometry applications in the food microbiology field are not limited to the cell counting even if this simple parameter is far to be easily obtained due to the presence of complex cell morphologies and aggregating structures among microorganisms associated to food matrixes. Beside the difficulties in microbial cell counting, other problems arise when the viability of microbial population has also to be measured. In this context, the viable but not cultivable fraction of the microbial population has important effect both in terms of quality and safety when pathogenic bacteria were analyzed. Flow cytometry has been also applied for the evaluation of several physiological parameters such as: the membrane potential, the membrane integrity, the intracellular pH, the activity of efflux systems involved in cell detoxification, the ability to form aggregates among cells belonging to different taxa. The presentation will be focused on flow cytometry applications in the fields of food microbiology and probiotics.

MONOCLONAL ANTIBODIES AND MULTIPLE MYELOMA

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The treatment landscape of patients affected with multiple myeloma (MM) is rapidly evolving. One of the most recent advances is represented by a targeted approach using monoclonal antibodies (MoAbs) directed against antigens expressed on the surface of MM plasma cells and other immune-competent cells. Of interest, available MoAbs destroy neoplastic cells mainly through the immune system and/or by inducing apoptosis. They have also demonstrated acceptable tolerability. So far, the clinically better investigated MoAbs in MM are daratumumab (dara) and elotuzumab (elo).
Dara is a fully-human MoAb directed against CD38, a transmembrane protein which is highly and uniformly expressed on MM cells and, at lower level, on other immune-competent cells, as well as in some non-hematopoietic tissues. Dara exerts: i) an immune-mediated activity through complement, macrophages and NK cells; ii) a direct, pro-apoptotic effect; iii) an immune-modulating action against T-reg and myeloid derived suppressor cells; iv) an inhibitory effect on CD38 metabolic activity.
In phase I-II trials of patients with relapsed-refractory MM (RRMM), dara as single agent has induced an overall response rate (ORR) of 31% (13% at least very good partial response or VGPR). Median duration of response was 7.6 months, that of overall survival (OS) 20 months This compared favorably with other treatments including proteasome inhibitors and IMIDs in the same setting of patients.
Recently updated results of two phase III studies have demonstrated that dara strongly synergizes with bortezomib plus dexamethasone (D-VD) or lenalidomide plus dexamethasone (D-LD) in patients with RRMM (ORR 84-93%, with 62-78% of at least VGPR and 77-76% of PFS at 12 and 18 months with D-VD and D-LD, respectively), thus significantly improving the effect of two-drug combinations (VD or LD). In particular, 67-63% reductions in the risk of disease progression or death were achieved using D-VD or D-LD, respectively. Benefits were obtained in absence of additional significant toxicities and regardless of previous treatments or cytogenetic risk, with promising, though still preliminary, effects on OS.
The most frequent adverse events for dara are infusion-related reactions (IRR), which occur in about half of patients, mainly during the first infusion(s). Grade > 3 IRR are, however, rare (< 3%). Thus, dara requires prolonged duration of first infusions, adequate dilution and appropriate pre- and post-medications. If adequately managed, IRR should do not represent, generally, a cause of dara definitive discontinuation. The possibility to administer dara s.c., in order to reduce time of infusion and IRR, has been recently reported.
CD-38 is present on the surface of red cells, so that dara may interfere with blood type characterization, inducing a durable false-positive Coombs' test; such an interference is not clinically relevant and blood products for transfusion can be identified for dara-treated patients by blood banks performing routine compatibility tests or by using genotyping. In this setting appropriate information must be given to blood bank, preferably before MoAb is started. Likewise, patients should carry a blood transfusion card indicating that they receive anti-CD38 MoAb therapy.
Dara is currently approved in Italy as monotherapy for the treatment of adult patients with RRMM, whose prior therapy included a proteasome inhibitor and an immunomodulatory agent and who have demonstrated disease progression on the last therapy.
Elotuzumab is a humanized, chimeric, immune-stimulatory MoAb that recognizes SLAM-F7, a cell surface "signalling lymphocyte activation molecule", which is highly expressed by MM and natural killer cells. Elo causes MM cell death i) directly activating natural killer cells (via EAT-2 specific protein), and ii) tagging for recognition of antibody-dependent cell-mediated cytotoxicity (ADCC) via CD16 mediated pathway.
Elo is not active as single agent in MM, but a phase III study has demonstrated that the combination of elo with lenalidomide and dexamethasone (E-RD) is significantly superior to simple RD, in terms of ORR (79 vs 66%, with 34 vs 29% of at least VGPR) and 3-yrs PFS (26 vs 18%), inducing 27% reduction in the risk of disease progression or death. Toxicity was acceptable and a lower rate of IRR with respect to dara was seen.
Elo is currently approved in Italy in combination with lenalidomide and dexamethasone for the treatment of RRMM after at least one prior therapy
Being dara and elo both MoAbs, perceived depth of response (in particular at immunofixation level) may be impacted by the presence of these drugs, with possible underestimation of complete responses and a possible premature determination of biochemical relapse.
Ongoing studies are exploring the role of other combinations with dara or elo in RRMM or as frontline therapy, including smoldering myeloma (SMM). Furthermore, there are several MoAbs that are under advanced clinical investigation in MM, such as further anti-CD38 molecules (Isatuximab and MOR202), check-point inhibitors (pembrulizumab, nivolumab), and others. MoAbs will be

probably a backbone that could really change the treatment paradigm in the next years and, hopefully, the future outcome of MM patients, with particular emphasis on long term immunological control of the disease.

IN FLUXO ANALYSIS: THE POWER OF REAL-TIME CYTOMETRY

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A very important feature of flow cytometry is the capability of providing kinetic data by determination in real time of changes in fluorescence or light scatter parameters of the cell population in study. Unlike other bulk real-time approaches, flow cytometry is based on the analysis of single cells, thus allowing simultaneous characterization of distinct subpopulations within heterogeneous cytomes. We propose the term "*in fluxo*" to highlight the special properties of data acquisition and result generation by real-time flow cytometry. The differential characteristic of *in fluxo* analysis would be that the kinetic plots are constructed by sequential sampling of different single particles that represent the behavior of the whole population at each time point. By means of appropriate gating, distinct particle subpopulations can be studied simultaneously. The biological process of interest evolves while the individual cells are examined in real time in the flow cytometer. Practically, the suspensions of cells or biological particles are studied *in fluxo* to reveal fast and transient processes. The variations in fluorescence- or scatter parameters are detected by means of kinetic plots, using time as a cytometric parameter. Because of the high sample rate (up to thousands of cells per second), the theoretical temporal resolution analysis of the *in fluxo* analysis may be close to the millisecond, while the duration of the continuous analysis may go from few seconds to hours and the total number of cells analyzed can be very high. While typically *in fluxo* analysis is aimed to assess the kinetics of extracellular fluorochrome uptake or intracellular fluorescence variations, the kinetic analysis of structural changes allows to determine secretion-associated degranulation, cell swelling associated to fluid uptake and loss of cellular integrity. *In fluxo* analysis has been mostly used for the study of cell signaling and cell activation, but in this presentation we will illustrate with our own data how it can be applied successfully to the integration of cellular processes in real-time Pharma-Tox and Flow Cytoenzymology.

THE GENOME AND EPIGENOME OF SPERM, A TRANSGENERATIONAL BRIDGE

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The cytological analysis of sperm is a consolidated tool to assess the fertility potential of an individual. Along the years, new molecular methods have greatly increased our capacity to analyse semen quality, especially in relation to its genetic integrity, thus allowing the evaluation of possible transgenerational effects. Nowadays, available tools include the measurement of DNA lesions and chromatin structural alterations, the assessment of aneuploidies, and even the detection of all *de novo* mutations occurred in the germline with respect to the somatic genome, by means of DNA sequencing. These tools allow to estimate with increasing sensitivity the impact of environmental factors on male reproductive health. To this aim, research approaches include laboratory experiments with animal models under controlled exposure conditions, as well as population studies under real exposure scenarios. The evolutionary conservation of spermatogenesis and the possibility to analyse the same target cell in both experimental animals and humans favour the integration of data obtained by different experimental approaches. More recently, the epigenomic dimension has enlarged the analytical space of sperm quality assessment. Sperm epigenomic biomarkers include DNA and chromatin chemical modifications (e.g. methylation) without impact on the base sequence, as well as changes of the content of small non coding, regulatory RNAs. The discoveries that such biomarkers can be modulated by environmental influences, and that changes are heritable, sometimes with phenotypic consequences on the progeny, open new perspectives to the study of human male infertility causes and of paternal contribution to the origin of multifactorial disease susceptibility.
This presentation is dedicated to the memory of our friend, mentor and colleague Marcello Spanò. With his wide vision, his thoughtful scientific rigour, and an encyclopedic culture he contributed creatively to the genetic and epigenetic characterization of male gamete and to the study of relationships between environment and male fertility.

ALLERGOLOGY AND FLOW CYTOMETRY: BASOPHIL ACTIVATION TEST

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In vitro diagnostic flow chart for allergic diseases has been based for many years almost exclusively on robust tests for the determination of total and specific IgE. In recent years, the introduction of molecular allergology increased the in vitro diagnostic opportunities through the evaluation of specific IgE directed to the single allergenic proteins responsible for the allergic reaction or for the cross-reactivity between pollens and foods. However, molecular allergology is mainly limited to clarify allergic reactions to inhalants, foods, insect venom, but not to drug reactions. Drug hypersensitivity reactions (DHRs), due to immunological mechanisms, represent about 15% of the so called Drug Adverse Reactions. DHRs can be mediated by different mechanisms; they can be immediate with mastocytes and basophils involvement or delayed primarily caused by T lymphocytes or by neutrophils or eosinophils. Basophil activation test (BAT) is a relatively new diagnostic tool, particularly for immediate drug reactions (angioedema, urticaria, gastrointestinal reactions and anaphylactic shock). BAT is based on the evaluation with flow cytometry of basophil activation markers like CD63, expressed on membrane only after activation or CD203c, constitutively expressed by basophils but with a higher number of molecules on the membrane after activation (1). Peripheral blood is pre-incubated with IL-3, in order to pre-activate circulating

patient's basophils, and then with different dilution of the suspected drugs. After the activation steps, cells are stained with specific monoclonal antibodies to surface activation markers. Gating strategy mostly used to select basophils among all the leukocytes (they are usually less than 1% of all leukocytes), is based on the expression of IgE on the membrane of basophils or on IL-3 receptor CD123 or eotaxin receptor CCR3.
BAT can also be used to evaluate inhalant or food reactions, particularly when routine tests offer contradictory results or are negative in the presence of a suggestive history (2). BAT can also be useful in insect venom diagnostic flow chart, to find the specific insect venom responsible for the reactions excluding cross-reactivity (3). BAT finds a particular application also during specific immunotherapy to mites or pollens, highlighting the decrease of reactivity towards the specific allergen.
Among drug reactions, those to beta-lactams are the ones for which BAT is more requested, but the test has been proved useful also in case of reactions to other antibiotics, local and general anesthetics, corticosteroids, and anti inflammatory drugs, in the most limited cases of reactions not due to inhibition of cyclooxygenase I (COX). Additives and colorants present in food and drugs can be tested too.
Although BAT procedure and flow cytometric acquision of the samples can be performed in every laboratory with the proper equipment, the methodology can present some difficulties and the knowledge of allergic reaction mechanisms, of basophil characteristics and of molecular allergology is mandatory to a correct interpretation of the results.

HUMAN NEOCENTROMERES AND CENTROMERE EVOLUTION

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Human clinical neocentromeres are perfectly functioning analphoid centromeres which emerge in ectopic chromosomal regions. Most of them arise in acentric, supernumerary chromosomal fragments whose mitotic survival is rescued by the neocentromere. These extrachromosomes result in phenotypic abnormalities that bring these patients into the clinical setting. Evolutionary studies have shown that the centromeres can reposition along the chromosome during evolution. Evolutionary new centromeres, also termed repositioned centromeres, were documented in a variety of eukaryotes. The relationship among "clinical" neocentromeres and evolutionary novel centromeres will be illustrated by analyzing the paradigmatic examples of chromosome 3, 6, 9, and 15.

MIGRATION AND ACTIVATION OF PHAGOCYTIC CELLS: NOVEL APPROACHES AND PATHWAYS

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Migration and activation of phagocytic cells represent critical steps for the establishment of immune responses. Upon inflammation, neutrophils leave the bone marrow, extravasate and migrate towards inflamed tissues following chemotactic gradients. Although the cytoplasm can quickly change consistence and form to allow cells to interact with and penetrate the endothelium, the deformation of the nucleus, the largest and stiffest cellular organelle, seems to be an awkward process.
Once in tissues, phagocytic cells exert their function consisting in pathogen recognition and ingestion and cytokine production. The signals that connect these two events – phagocytosis and cytokine response – are still unclear.
Exploiting in vivo models, flow cytometry, confocal microscopy, live cell imaging and microfluidic devices, we investigate novel signaling pathways involved in the orchestration of cell migration, phagocytosis and cytokine production.

Allergology

AN ALTERNATIVE CYTOFLUORIMETRIC METHOD FOR EVALUATING IN VITRO SKIN SENSITIZATION

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In recent years, many progresses have been done in the development of non-animal tests to assess skin sensitization process. One of the alternative assay accepted at the OECD (OECD TG 442E) is the human cell line activation test (h-CLAT), which measures the variation in the expression of CD54 and CD86 surface markers induced by sensitizing substances in THP-1 cell line monitored by means of flow cytometric analysis. Antigens are detected via specific monoclonal antibodies labelled with fluorochromes, such as Fluoresceine-isothiocyanate (FITC). Unfortunately, the level of cell auto-fluorescence overlaps the emitted fluorescence inducing an overestimation of the generated fluorescence signals. This event generate the need of troubleshooting in data analysis and interpretation. The aim of this study was to find an alternative parameter (such as cell shape and morphology) more sensitive than fluorescence to evaluate skin sensitization potency of chemicals.
For this purpose, THP-1 cells were seeded at density of $0.2x10^6$ cells/ml in 75 cm2 culture flask and cultured for 48 hours. Then cells were transferred in a 24 well plate ($1x10^6$ cells/well) and treated with different allergens and non-allergens substances. For control analysis, a set of cells wasn't exposed to any chemical and has been maintained in culture medium. After a 24 hours period of incubation, cells were washed twice in phosphate-buffered saline and flow cytometric analysis was performed with a Partec Cy-Flow-Space cytometer. We observed a significant change in the forward scatter (FSC) of cells treated with skin sensitizing substances. No changes were observed in control cells and in cells treated with non-sensitizers.
Our results suggest that sensitizing chemicals, like 2,4 dinitrochlorobenzene (DNCB), are able to induce morphological changes in THP-1 cells, as demonstrated by variations in the FSC signal. For these considerations, this test system can be used as a sensitive, fast and low-cost method for the analysis of sensitizing substances allowing to discriminate between sensitizers and non-sensitizers.

THE CONTRIBUTION OF FLOW CYTOMETRY IN THE DEVELOPMENT OF A NEW IN VITRO SKIN SENSITISATION TEST (HUMAN CELL LINE ACTIVATION TEST H-CLAT)

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Background. Legal frameworks regulate the safety assessment of chemical substances; particularly, the Regulation (EC) No 1223/2009 on cosmetic products takes into account all the characteristics of cosmetic ingredients highlighting an important toxicological endpoint such as skin sensitisation. According to the Directive 2010/63/EU (protection of animals used for scientific purposes), alternative methods to test skin sensitisation have been developing and validating for 17 years, such as the new *in vitro* procedure human Cell Line Activation Test (h-CLAT). The aim of this work was to classify two sun products as skin sensitiser (that will lead to an allergic response following skin contact) or not, applying the h-CLAT according to the recent Organisation for Economic Co-operation and Development (OECD) Guideline. **Methods**. Two concentrations, obtained by cell viability test, were tested for each sun product: SPF 30 (100, 500 µg/ml) and SPF 50+ (4, 20 µg/ml). The h-CLAT procedure included a preliminary reactivity check to establish the possibility to quantify changes in the expression of cell surface markers, using flow cytometry. The changes were associated with the process of activation of monocytes and CD86 and CD54, in the human monocytic leukaemia cell line THP-1, following exposure to potential sensitisers, including positive control (such as nickel sulfate). **Results**. The results were reported not only as Relative Fluorescence Intensity (RFI) of CD86/CD54 for positive control cells and potential sensitiser-treated cells, but also as Forward Scatter (FSC) and Side Scatter (SSC) signals, which could play an important analytical role being related to cell morphology. For CD86/CD54 expression measurement, each product was tested in at least two independent runs to derive a single POSITIVE prediction: the RFI of CD86 ≥ 150% and the RFI of CD54 ≥ 200%, at any tested concentration. Therefore, according to the obtained fluorescence data, we classified the sun product SPF 30 as sensitizer and the sun product SPF 50+ as non-sensitiser. Moreover, the observation of cell morphological changes, which correlated with cell damages, confirmed these results. **Conclusions**. The new *in vitro* procedure h-CLAT was recommended to be used to support the discrimination between sensitisers and non-sensitisers for the purpose of hazard classification and labelling. Furthermore, the monitoring of cell morphology by means of a FSC/SSC correlation proved to be important to explain and support fluorescence results.

UTILITY OF BASOPHIL ACTIVATION TEST (BAT) IN DIAGNOSIS OF EGG- ALLERGY IN CHILDREN

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Background- Egg allergy is one of the most common IgE-mediated food allergies in children, affecting approximately 1,2 % of children under 2 years of age. However, more than 50% of children would outgrow this condition by the age of 5. Double-blind placebo-controlled oral food challenge currently represents the gold standard for food allergies diagnosis. Nevertheless, food challenges may be expensive, time-consuming and potentially dangerous. Hence, a reliable, non-invasive and safe diagnostic method is required.

Objective-To evaluate the utility of the basophil activation test (BAT) in the diagnostic work-up of egg allergic children.
Methods- 100 children (mean age at oral food challenge (OFC) 4 years) with suggestive history for egg allergy were enrolled in our Department of Pediatrics. Patients underwent clinical evaluation, skin prick tests (SPTs), prick by prick (PbP) with egg white and yolk, specific-IgE to egg and its components (ovomucoid, ovoalbumin, ovotransferrin and lysozyme), OFC and BAT (performed by flow cytometry). Statistical analysis was executed using the *R statistical software version 2013*. ROC curves to identify optimal cut-off points for the wheal diameter obtained by SPTs and the one obtained by PBPs, s-IgE levels and basophilic activation following stimulus were built. BAT sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV) were determined.
Results- ROC curve analysis for basophilic activation following stimulation with egg white and yolk provided 82% accuracy, 60% sensitivity, 91% specificity, 16% PPV, 98.7% NPV, considering a threshold of 31.76%. Therefore BAT showed to be less sensitive, but more specific than SPTs, PbP, sIgEs in our cohort.
Conclusions- BAT proved to be particularly useful and to be superior in cases in which specialists could not accurately diagnose egg allergy with the other diagnostic tests. Hypothetically, this may justify the use of a 2-step diagnostic approach in which BAT is performed only after equivocal SPTs, PbP or sIgE, allowing the allergologist to achieve a more accurate and cost-effective diagnosis. Nevertheless, if BAT results are ambiguous or negatives, the OFC should be performed. Further studies are needed to determine the role of BAT as a diagnostic *in vitro* test in food allergy work-up.

USE OF BASOPHILE ACTIVATION TEST IN CHILDREN WITH SUSPICION OF ALLERGY TO BETA- LACTAMS

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Background- The allergy work up for the diagnosis of hypersensitivity to B-Lactams drugs currently requires the execution of correct tests, like skin prick test, intradermal test and oral provocation test, which require an important commitment of resources, in terms of time and money, and a stressful process for young patients. The Basophile activation test (BAT) has been proposed as a new diagnostic tool in the allergic work up for the diagnoses of B-Lactams IgE-mediated hypersensitivity in children and adults.
Material and Methods- 55 Childrens, with history of immediate (13) or delayed (42) reactions to Amoxicillin, Amoxicillin-Clavulanate and various Cephalosporines where investigated performing BAT, skin prick test, intradermal test and oral provocation test for the suspect of an IgE-mediated hypersensitivity to this drugs. Tests were performed in Pavia, in pediatrics of IRCCS Policlinico San Matteo, during years 2015 and 2016.
Results- BAT was positive in 10 patients, all with delayed reactions, but only one of these patients (10%) had a positive oral provocation test result. The others 4 patients with a positive drug allergy work up had a negative BAT result. The Negative Predictive Value (NPV) is 93%
Conclusions- BAT does not seem to be a useful diagnostic exam in an allergy work-up for the diagnoses **of** hypersensitivity to B-Lactams in children for a low sensitivity, but it has a good NPV. The research of new diagnostic tools aimed to simplify the allergy work up of drugs hypersensitivity is an important field of study and new findings would definitely have a big impact on current diagnostic processes.

BASOPHIL ACTIVATION TEST FOR STAPHYLOCOCCUS ENTEROTOXINS IN SEVERE ASTHMATIC PATIENTS

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Introduction: Recent studies suggest that IgE antibodies to Staphylococcus aureus enterotoxin represent a risk factor for severe asthma even in asthmatic patients classically considered nonatopic. Staphylococcus aureus enterotoxins can stimulate specific IgE responses moreover, acting as superantigens, they can also promote a polyclonal IgE response, airway inflammation, and bronchial hyperresponsiveness. In comparison with the measurement of serum specific IgE, the Basophil Activation Test (BAT) can give more relevant results because it measures only functional IgE capable of binding and activating basophils. BAT for staphylococcus enterotoxins has never been performed until to now.
Methods: We recruited 35 patients with severe asthma treated according to GINA guidelines. They were tested for skin prick test to common aeroallergens. Total and specific IgE to staphylococcus enterotoxins (ImmunoCAP) were measured. Nasal swabs and sputum cultures were obtained. Basophil activation tests (BAT) using CD203c expression was done after stimulation with different concentrations of enterotoxins A, B, and toxic shock syndrome toxin (Sigma).
Results: BAT for at least one of Staphylococcus aureus enterotoxin was positive in 13 among 35 severe asthmatic patients (37%) while specific IgE to staphylococcus enterotoxins were detected in 18 patients (54%). The percentage of positive BAT for SA enterotoxins was higher in non-atopic than in atopic severe asthmatic patients (42 % vs 34%). No relationship was observed between staphylococcus aureus nasal colonization and the presence of IgE and/or the positivity of basophil activation test for staphylococcus enterotoxins.
Conclusions: In this study, we demonstrate the involvement of specific IgE mechanisms in severe asthmatic patients sensitised to staphylococcus enterotoxins. The potential benefit of anti-IgE as well as local (intranasal) or general antibiotic therapy in this subgroup has to be investigated.

DIAGNOSIS OF HYMENOPTERA VENOM ALLERGY: CORRELATION WITH CLINICAL HISTORY, SKIN TEST, SPECIFIC IGE AND BASOPHIL ACTIVATION TEST (BAT)

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INTRODUCTION: Hymenoptera stings are sometimes

fatal in venom-allergic patients and correct diagnosis is a prerequisite for effective and potentially life-saving management, i.e. specific venom immunotherapy (VIT). Currently, diagnosis of venom anaphylaxis generally relies upon an evocative history corroborated by positive venom skin tests (VST) and venom-specific Hymenoptera IgE (VH-IgE) level. Cellular in vitro tests such as the CD63-based basophil activation test (BAT) have been successfully used to diagnose hymenoptera venom sensitization in adult patients.
METHODS: Thirteen patients (5 male, 8 female; 10-63 years old, mean 42,15 years) with a definite history of allergy to yellow jacket venom and/or honey bee and/or paper wasp venom and/or European hornet venom were enrolled. Tests were performed with the FACSCanto flow cytometer by using double-labeling with phycoerythrin (anti-CCR3-PE) and anti-CD63-FITC. The cut-off for a positive BAT was 10%. The VH-IgE levels were determined by using the CAP-FEIA method. The sensitivities and specificities for the BAT and VH-IgE levels were calculated against the results of the VST.
RESULTS: A total of 22 tests were performed. Of the 15 positive VST–results, 10 and 13 were correctly assigned by BAT and VH-IgE test, respectively. Among the 7 negative VST–results, 7 and 1 were correctly detected as negative by BAT and VH-IgE test, respectively. Using the last test, 5 gave a no interpretable result. Sensitivities of the BAT and VH-IgE test were of 66.7% (95% C.I: 38.38% to 88.18%) and 86.7% (95% CI: 59.54% to 99.34%), respectively, while specificities were of 100% (95% CI: 59.04% to 100.00%), and 14.29% (95% CI: 0.36% to 57.87%), respectively. When the results of IgE specific they were no interpretable, the BAT has been able to correctly discriminate 80% of the cases.
CONCLUSIONS: Quantitation of basophil activation by CD63 expression is a valuable in vitro method for diagnosis of allergy to hymenopteran venoms. Being used in conjunction with measurement of IgE levels, the *in vitro* diagnosis reaches an accuracy comparable to the *in vivo* testing. Finally, BAT test improves the discrimination between doubtful results of IgE specific.

UTILITY OF BASOPHIL ACTIVATION TEST (BAT) IN ALLERGEN-SPECIFIC IMMUNOTHERAPY IN CHILDREN

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Background: Allergen immunotherapy (AIT) is recommended in children with allergic rhinitis with/without allergic asthma, with an evidence of specific IgE-sensitization towards clinically relevant inhalant allergens. Long-term studies provided evidence that AIT can also prevent the onset of asthma and of new sensitizations.
Methods: In this study we included all children referring to the Paediatric Clinic of the Foundation IRCCS Policlinico San Matteo of Pavia sensitized to grass or dust mites and treated with an allergen-specific immunotherapy. The purpose of our study was to evaluate the use of Basophile Activation Test (BAT) as a biomarker in the follow-up of immunotherapy and allergy desensitization. BAT values were determined before the treatment and after 1 year with 3 different dilutions (7ng, 70ng, 700ng) of allergens.
Results: In the 5 paediatrics patients that began an allergy immunotherapy we observed that after 1 year of treatment all patients referred an improvement of symptoms and BAT values showed a decrease of percentage of CCR3+ CD63+ (%) in each dilution tested: medium value of CD63+ decreased from 25% to 14% in 7ng dilution, from 49% to 21% in 70ng dilution and from 58% to 43% in 700ng dilution.
Conclusions: BAT's values are associated with an improvement of clinical conditions and symptoms. The use of BAT in children treated with allergy immunotherapy could be suggested as an useful biomarker to evaluate the efficacy of desensitizing treatment. We can't exclude that in the future BAT could be used in monitoring of biologic efficacy and to decide in a safer way to continue with the treatment for 4-5 years or to interrupt it.

BASOPHIL ACTIVATION TEST IN THE DIAGNOSIS OF DRUG HYPERSENSITIVITY: A CLINICAL EXPERENCE

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Introduction: Drug hypersensitivity reactions (DHRs), due to immunological mechanisms, represent about 15% of the so called drug adverse reactions (DARs). Diagnosis of DARs is based both on clinical history, and in vitro/vivo tests. The basophil activation test (BAT), which evaluates basophil activation markers with flow cytometry, can be a supplementary tool for drug allergy diagnosis.
Subjects and methods: We evaluated 204 outpatients reporting DHRs. Serum specific IgE were determined (ImmunoCAP System, Sweden) and skin prick test (SPT) were performed when appropriate. BAT was performed immediately after blood sampling. Fifty µl of peripheral heparinized blood were pre-incubated with IL-3 and then with drugs freshly prepared from the injectable solution commercially available. The expression of CD63 was determined with flow cytometry (FacScalibur, BD Biosciences). A range of 400-1000 basophils was considered suitable for the analysis. The test was positive when CD63 expression was >5% and the stimulation index (SI=ratio % CD63 with drug / % CD63 with wash buffer) was > 2.
Challenge test: patients who reported mild -severe reactions and those with discrepancy between clinical history and BAT underwent a challenge test.
Results: 1652 BAT were performed in the enrolled patients. Fifty-three % of the positive tests were due to antibiotics: penicillins (amoxicillin 21%), quinolones (21%) and cephalosporins (17%), whilst macrolides were less involved (7%). Twenty-five % of subjects had a positive BAT for NSAD: paracetamol was responsible for 45% of the reactions among the NSAD, followed by ketoprofen (12%), ibuprofen (10%), ASA (10%) piroxicam 8%, nimesulide 4%, and diclofenac 5%.
Eighty subjects were challenged with the suspected drugs (69 for antibiotics and 11 for NSAD). We observed a low sensitivity of challenge test (29 %) and high specificity (95%) for all the tested antibiotics. For the suspect-

ed reactions to penicillins, the highest contribute in terms of sensitivity was due to BAT and specific IgE (65%), and both the in vitro tests supported 94% of diagnosis. Only one subject with negative BAT and sIgE had a positive cutaneous test for penicillins.
Conclusion: BAT can well discriminate DARs, whilst only more critical cases require an integrated evaluations and more complex clinical examinations. It is relevant that the concordance of anamnesis and in vitro tests reduces the need of challenge tests limiting them to selected cases.

BASOPHIL ACTIVATION TEST (BAT) IN CLINICAL PRACTICE: PRELIMINARY DATA

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Background: *in vivo* drug tolerance test are still considered as gold standard test to diagnose allergies to drugs. Anyway, these procedures can not be considered risk-free test for patients. Indeed, the patient may develop a severe allergic reaction during the execution of the test.
BAT has been proposed, as a possible risk-free assay for the diagnosis of immediate-type allergic reaction and hypersensitivities against suspected allergens. The assay is based on the *in vitro* detection of basophil activation (increase of CD63 marker) by flow cytometry.
The aim of this study is to evaluate the utility of BAT in the diagnosis of allergies in adults.
Material and methods: Ten adults, with clinical history of allergies, underwent both *in vivo* test (skin prick test, oral drug tolerance test) and *in vitro* test (BAT). Different type of antibiotics (Doxycycline, Clarithromycin, Ciprofloxacin, Levofloxacin and Ceftriaxone) were tested. One patient was excluded because had a tardive reaction. The results were analysed to evaluate the concordance between the two test-
Results: among the 9 patients evaluated, all of them had a negative response to allergens when underwent both *in vitro* and *in vivo* test.
Conclusions: although, a small number of patient has been studied, the preliminary data show that the basophil activation test should be an useful and safer tool for *in vitro* diagnosis of immediate allergies.

Environmental Sciences and Toxicology

ANALYSIS OF GENETIC STABILITY OF MICROPROPAGATED PLANTS OF PYRETHRUM BY FLOW CYTOMETRY

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Keywords: pyrethrins, micropropagation, true-to-type, ploidy stability

Pyrethrum (Chrysanthemum cinerariaefolium L. = Tanacetum cinerariaefolium (Trev.) Schultz-Bip.) is a perennial herbaceous plant belonging to the Asteraceae family. It is largely grown for the extraction of pyrethrins, natural insecticides and insect repellents, which are found in the flower head and extracted primarily by dried explants. Hence, pyrethrum is addressed to chemical industry for the manufacturing of natural pyrethrins-based items, used in organic farming. A fast propagation of pyrethrum clones it is of most importance for rising the amount of piretrins which are needed by industry to fulfill the worldwide demand for natural insecticides and generate the large quantities of phytomass with standard chemical properties. We have established an efficient protocol for in vitro micropropagation of pyrethrum and for the acclimatization of micropropagated plants in field. Well developed plantlets that achieved the optimal vigour conditions were transferred in the field, showing a good suitability to field conditions with 60% of survivals.
Plantlets derived from in vitro culture may exhibit genetic variability, namely somaclonal variations. In the present study, we estimated the ploidy level of micropropagated plants of pyrethrum transferred in field (ex vitro conditions) by flow cytometry analysis.
Flow cytometric analysis of leaf nuclei in suspension allowed to a fast and precise characterization of DNA content (ploidy level) in micropropagated plants. A large flow cytometric screening for major ploidy changes revealed no differences among the regenerated plants and between them and the mother true-to-type plant.

DETECTING MICROBES IN SPACE WATERS: NEW INSIGHTS BY FLOW CYTOMETRY

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Aquatic microbes are primary constituents of all known humid areas and water sources, including those found aboard the International Space Station, future human space exploration vehicles and planetary outposts. The complex

treatment systems providing safe drinking waters during spaceflights are inherently prone to biofilm formation and microbiological contamination that can be attenuated but never eliminated. It has been further noted that both human physiology and microbial communities adapt to space conditions, with microbial pathogenicity increasing under microgravity. Thus, space exploration requires the development of rapid and safe approaches for preventing, monitoring and controlling biocontamination within human confined environments. Here we aimed to (i) cross-validate reliable measurement methods to assess microbial cell densities, and (ii) provide new insights on microbial community composition, either in terrestrial waters with different quality (from potable to wastewaters) or in drinking space waters (after long-term exposure to microgravity). An array of current methodologies were applied, including plate cultivation, ATP-metry, flow cytometry, and qPCR. In the framework of the H2020 project BIOWYSE, our results allowed identifying alternative standards of microbiological quality, thus providing the backbone dataset to develop and test innovative prevention, monitoring and mitigation modules to face microbial contamination risks for space and Earth-based water applications.
Keywords: Aquatic microbial communities, human confined habitats, International Space Station (ISS), cell quantification methods, space contamination.

ANNUAL VARIATION OF PLANKTONIC BACTERIAL AND VIRAL ABUNDANCE AT A COASTAL SITE IN THE GULF OF NAPLES

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Viruses represent the most abundant planktonic community in aquatic environments and thus are involved in ecological processes as they influence carbon and energy flows among the trophic levels through control of bacterial populations by viral lysis and nutrient turnover. The abundance and distribution of bacteria and virus and their ratio were investigated during 2014 at the Long Term Ecological Research Station MareChiara (MC), in the Gulf of Naples. Bacterial and viral abundances were estimated by flow cytometry. Heterotrophic bacteria were homogeneously distributed along the water column during winter as a consequence of seasonal mixing, but concentrated at surface during the other periods. The highest concentrations were recorded at surface in spring April-May (1.88 x 10^6 cell ml^{-1}, 1.67 x10^6 cell ml^{-1}) and in summer (1.11 x 10^6 cell ml^{-1} and 8.73 x 10^5 cell ml^{-1} in July and September, respectively). This distribution may be explained by the local terrestrial inputs in terms of organic matter and nutrients used by bacteria for their growth. Virus highest concentrations were observed in spring and summer (9.35 x 10^6 cell ml^{-1}, 1.56 x 10^7 cell ml^{-1}, 1.18 x 107 cell ml^{-1} May, July and August respectively), just after peaks of bacterial concentrations, suggesting a release of viral particles following bacterial lysis.These results support the hypothesis that viruses depend on the bacterial host populations at sea and this is supported by the statistically significant correlation ($p < 0.0001$) between total heterotrophic bacteria and viruses. This also suggests that when bacteria reach very high concentrations they attract more viruses that, by their lithic action, control bacterial biomass and dynamics, elaborated as the “kill the winner” strategy. This is represented by the Virus to Bacteria Ratio (VBR). The influence of virus on bacterial production and dynamics is closely linked to the trophic conditions and productivity of the ecosystem and viral activity potentially helps to maintaining high levels of biomass and productivity in the system. This work represents the first study on the annual viral activity and virus-bacteria interaction in the Gulf of Naples.

GREEN SILVER NANOPARTICLES WITH PECTIN: AN ANALYSIS OF ANTIBACTERIAL AND ANTI-BIOFILM PROPERTY

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Introduction: Synthesis, biomedical properties of silver nanoparticles (AgNP) and their use as additives for wound-healing biomaterials endowed with antibacterial properties have been extensively investigated. A key factor for biocompatibility is the amount of Ag+ ion released by AgNP.
Biocompatible reductant agents and narrow dimensional distribution, a typical bottleneck of many AgNP synthesis procedures, are also required. Here, we report on an efficient, simple, green synthesis method of AgNP with citrus peel pectin (p-AgNP), used both as a reductant and coating agent, and their excellent antibacterial properties.
Material and methods: Pectin from citrus peel (0.5%,1%,2%) was dissolved at 60°C. Upon cooling, $AgNO_3$ solution was added to a final concentration of 1mM and 0.5M NaOH was added. Stirring was continued for 12/24 hours. *E. coli* PHL628 and *S. epidermidis* RP62A were used as model Gram – and + strains, respectively. Minimum Inhibitory Concentration (MIC) was determined in planktonic condition, as well as effect of p-AgNP was analyzed before and after biofilm formation. 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium-bromide (MTT) test was used to assess viability of bacteria. Confocal Laser Scanning Microscopy observations on bacterial biofilms were also performed.
Results and discussion: The new fast synthesis method provided an almost complete reduction of Ag+ to Ag0. The generated AgNPs displayed excellent long term stability, narrow dimensional distribution and low Ag+ release. Despite this, excellent MIC values were reported for both strains, close to or lower than $AgNO_3$ ones. This effect is likely due to the weak interaction between p-AgNP pectin coating and bacterial surface. Similarly good properties were reported for their activity on biofilms in the two different experimental settings analyzed. In both planktonic and biofilm conditions, *E. coli* showed to be more affected, owing to its documented higher Ag+ sensitivity compared to *S. epidermidis*.
Conclusion: We have generated stable, dimensionally uniform AgNP, with pectin being both reducing agent during synthesis and coating of AgNPs themselves. Our p-AgNP are the best compromise between good antibacterial and anti-biofilm effect and possible cytocompatibility issues, the former normally requiring sustained Ag+ release, that is detrimental for human applications because of cytotoxicity.

DISCRIMINATION BETWEEN BACTERIA, PICOCYANOBACTERIA, SULFUR-BACTERIA AND EUKARYOTES BY FLOW CYTOMETRY IN FIVE VOLCANIC LAKES FROM THE AZORES ARCHIPELAGO (PORTUGAL)

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São Miguel Island (Azores Archipelago, Portugal) hosts five volcanic lakes: Sete Cidades, Santiago,
Fogo, Congro and Furnas. Due to their peculiar thermal characteristics, they can be defined as atelomictic with partial stratification and destratification on a daily basis. This thermal regime is peculiar of tropical lakes and can influence lake functioning from both the chemical and biological point of view, as the extension of the epilimnion and metalimnion may vary. In summer, the lakes are stratified with a significant concentration of dissolved gas in the anoxic hypolimnion. These multilayer lakes have a dynamic structure and the lacustrine microbial communities are not easily distinguished. We measured different chemical and physical parameters and sampled top and bottom water from the five lakes to carry out a first survey, characterizing the microbial population by flow cytometer. We found marked differences of the microbial populations of the five lakes. Their composition revealed interesting autotrophic microorganisms and generally abundant bacterial population (around or above 5 xl06 cells mL-1). Picocyanobacteria were present with phycoerythrinrich (PE) or phycocyanin-rich (PC) cells depending on the prevalent phycobiliproteins. PEs were abundant in Lake Sete Cidades, whereas PCs and eukaryotes prevailed in Lake Congro. Lake Furnas, Congro and Sete Cidades were characterized by the presence of Purple Sulfur Bacteria (PSB) in the anoxic bottom, visible in the cytograms as a cloud, with a strong signal in FL4 due to their pigments.
The five lakes had low, though significant CH4 concentrations at lake bottom, gradually declining in the upper layer. We hypothesized that the methane can likely be metabolized by sulfate reducing bacteria to produce H2S, successively used by PSB as electron donor to perform photosynthesis in the anoxic illuminated layer. They can be purple or green sulfur bacteria and differ in size and in S storage, as well as in pigment composition. These findings indicate that biogeochemical processes can significantly develop in volcanic tropical lakes and create micro environments harboring different microbial community.

DEVELOPMENT OF SINGLE CHROMOSOME GENOMICS FOR PLANTS

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Key words: chromosome genomics, haplotype phasing, flow sorting, MDA amplification

Nuclear genomes of many plant species are large due to high repeat content and their analysis is hampered by DNA sequence redundancy - in many species also due to polyploidy. Genome mapping and sequencing can be simplified by dissecting the genomes into their natural subunits - chromosomes. Flow cytometry is particularly useful for chromosome isolation as large numbers of chromosomes can be purified, their DNA is intact and suitable for a number of applications in molecular biology and genomics. However, in many species, discrimination of individual chromosomes is hampered due to their similar size and DNA content, which renders them undistinguishable on a flow karyotype. To overcome this limitation a method for preparation of DNA from single copies of chromosome was developed. Each individual copy of a chromosome is amplified about million-fold to obtain microgram quantities of chromosome-derived DNA that is suitable for various downstream applications, including next-generation sequencing. This approach makes it possible to identify genic sequences on particular chromosomes, develop chromosome-specific DNA markers, verify assignment of DNA sequence contigs to individual pseudomolecules, and validate whole-genome assemblies. The protocol expands the potential of chromosome genomics, which may now be applied to any plant species from which chromosome samples suitable for flow cytometry can be prepared, and opens new avenues for studies on chromosome structural heterozygosity and haplotype phasing in plants. This work has been supported by the National Program of Sustainability (grant award LO 2014).

REAL-TIME RAPID ASSESSMENT OF MARINE WATER QUALITY BY AUTOMATED HIGH-FREQUENCY FLOW CYTOMETRY

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Marine microbes are highly dynamic and react very fast to variations in ecosystem properties so as to acute pollution or meteorological events. In order to catch these reactions and to establish their causes, high-frequency accurate measurements in time and space are needed. In environmental sciences, automated flow cytometry has been used for autofluorescent microbes and for heterotrophic bacteria after staining. We have combined these two approaches by using the CytoSense (Cytobuoy bv) for the online analysis of phototrophic microbes and the onCyt automated flow cytometer (onCyt Microbiology AG), connected to the on-board pump of an oceanographic vessel while sailing, demonstrating that high spatial resolution monitoring of microbes distribution at surface is feasible and provides useful first-hand information that can be used as a rapid and real-time indication of water quality. The system was also connected to online probes of temperature, conductivity and fluorescence, so to relate the observed microbial distribution to environmental factors. During the cruise, several low-salinity spots were observed, marking terrestrial discharges of urban, industrial, and agricultural origin. One of these spots was located in front of an urban pipe discharging untreated civil waters. In this spot we also observed the highest heterotrophic bacterial and the lowest nanoplankton concentrations, suggesting favourable conditions for the growth of heterotrophs and smallest microbes, able to

thrive on organic carbon. The high bacterial concentrations detected probably represent a marker of altered ecosystem conditions, and potential represent a threat for human health, if bearing pathogens. This hypothesis needs to be tested with further appropriate methods such as cultivation or molecular probes. The system used in this study proves to be useful for real-time monitoring of water quality with very little supervision and as a first, generic assessment of potential environmental hazard. Microbial communities, in turn, appear to be useful markers of changed environmental conditions, that need to be considered and included in water quality assessments such as those sought by the European Directive 2008/56/EC, which aims at reaching a Good Environmental Status (GES) for all European waters within 2020.

USE OF NON-INVASIVE EXALED BREATH CONDENSATE (EBC) TO EVALUATE BIOMARKERS OF EXPOSURE AND GENOTOXIC/OXIDATIVE EFFECT IN CHROME-PLATING WORKERS

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Toxic metals are measurable in EBC, allowing the possibility to quantify the lung tissue dose of metals occurring in occupational settings and consequent pulmonary effects. Inhalation of Cr(VI) can induce genotoxic/oxidative and inflammatory effects on the respiratory tract. The aim of the study was to assess the sensitivity of EBC to document exposure to carcinogenic Cr(VI) and its early genotoxic/oxidative effects. We studied 36 workers using chromate-based paints. Environmental Cr and Cr VI exposure was monitored by personal air sampling and HPLC-ICP-MS. Biological monitoring included urinary Cr measured by Electrothermal atomic absorption spectrometry (ETAAS) and expressed as a function of creatinine excretion. In EBC total Cr level (by ETAAS), and biomarkers of oxidative stress, such as H2O2, malondialdehyde (MDA), H-NE, 8 isoprostane (either by LC MS/MS or ELISA kit) were measured. Moreover, the ability of EBC to induce direct/oxidative DNA damage by Fpg-comet test on human pulmonary cells (A549) was evaluated. Environmental total Cr and Cr(VI) were far below TLV. Urinary mean Cr level was 0.18 µg/g creatinine, without differences between pre- and end-shift sampling time. Total Cr in EBC was in the range (0.03-0.78µg/L) with no differences between pre- and end-shift samples. The biomarkers of oxidative stress MDA and HNE showed slight differences in EBC between beginning and end working week. Fpg comet on cells exposed to EBC showed in 14/36 (38.9%) cases a detectable DNA damage induction (about %DNAtail two fold increase vs control unexposed cells) correlating in 7 cases with higher EBC Cr levels and environmental total Cr/Cr (VI) and urinary Cr levels, and in 4 cases with higher levels of isoprostane (3/4) or H_2O_2 (1/4). The results show that despite the very low Cr exposure, total Cr is detectable in EBC of chrome plating workers, and that EBC levels of biomarkers of oxidative stress (MDA and HNE) are slightly increased at the end of working week. Fpg comet on cells exposed to EBC showed detectable direct DNA damage correlated with Cr exposure and EBC biomarkers of oxidative stress confirming the high sensitivity and ability of this assay to detect early and still reparable DNA damage. Our results suggest the use of not invasive EBC and sensitive Fpg comet to evaluate exposure and early effects at target organ in occupational metal industry.

EFFECTS OF DIFFERENT ENVIRONMENTAL VIBRIO STRAINS ON FUNCTIONAL PARAMETERS OF MUSSEL HEMOCYTES

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Marine bivalves are exposed to a large number of bacteria and the hemocytes are responsible for cell-mediated immunity against infections.
The aim of this work is study the short-term responses of hemocytes from *Mytilus galloprovincialis* to different Vibrio species isolated from environmental samples.
In order to do this *in vitro* experiments were conducted and were evaluated lysosomal membrane destabilisation, extracellular lysozyme release, oxyradical production and nitrite accumulation. Furthermore the induction of apoptotic processes were investigated by flow cytometry using Annexin V-FITC and Propidium Iodide.
Results evidence different responses depending on concentration and time exposure of hemocytes towards different Vibrio strains: *V. parahaemolyticus* Conero, *V. alginolyticus* 1513 and *V. vulnificus* 509. *V. parahaemolyticu*s ATCC 43996.
All vibrios induced an almost immediate release of lysosomal hydrolytic enzymes even if in a distinct time course was observed with different vibrio species and strains.
The FCM results show that *V.parahaemolyticus* Conero did not induce apoptotic processes, whereas V. vulnificus induced a significant increase in the percentage of Annexin V positive cells.
Understanding the relationships between *Mytilus* hemocytes and potentially harmful vibrio species contribute to predict their potential effects on the health status of natural and farmed mussel populations and consequent ecological and economic impacts.

FPG-COMET ASSAY AND MICRONUCLEUS TEST TO EVALUATE THE POTENTIAL GENOTOXICITY OF TWO TITANIUM DIOXIDE NPS ON HUMAN BRONCHIAL CELLS

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The widespread production and use of titanium dioxide nanoparticles (TiO_2NPs) in consumer products (cosmetics and food additives) and industrial and medical appli-

cations highlights the importance of identify reliable methods to assess their toxicity. Potential genotoxicity is among the most critical health risks. We applied Fpg comet and cytokinesis block micronucleus (CBMN) assays on human bronchial BEAS-2B cells, to test genotoxicity of two differently sized anatase TiO_2NPs obtained by JRC (NM100 size 50-100 nm and NM101 size 5-8 nm). NM characterization was performed by TEM analysis and DLS. The cells were treated with 0.1, 1, 10 and 100µg/ml TiO_2NPs for 24h (Fpg comet assay) and for 48h (CBMN) to detect direct/oxidative DNA damage and MN induction respectively. Trypan blue and ViaCount cytofluorimetric assays were used for cytotoxicity evaluation. NM100 resulted better dispersed than NM101 in water/BSA and in culture medium at the highest concentration while at 10 µg/ml both titania showed agglomerate sizes around 300-500 nm. No cytotoxicity was found by trypan blue. Slight cell viability reduction (about 85% of control for both NM) with slightly higher apoptosis for NM101 and death for NM100 was observed by ViaCount assay only at 100µg/ml. Fpg-comet assay showed for NM100 direct DNA damage at the higher concentrations reaching 4.08 fold the %DNA in tail control value at 100µg/ml and an induction of oxidative DNA damage at 10 µg/ml. For NM101 increase in direct DNA damage reaching a plateau at 10 and 100 µg/ml (about 3.80 fold the control) and a significant induction of oxidative DNA damage at the highest concentration were found. In the CBMN assay, no induction of MN and no reduction of replication index were found confirming the low cytotoxicity observed by the other assays. The findings show low cytotoxicity but genotoxic effects in terms of DNA strand breaks at 10 and 100 µg/ml for both TiO2NPs and oxidative DNA damage at the highest concentration of NM101 probably linked to its smaller size, higher cell uptake and agglomeration tendency and capacity to induce ROS. The lack of MN is probably due to the fact that CBMN assay is able to detect genetic lesions different from those detected by comet assay. The study show the higher sensitivity of Fpg comet assay to evidence early genotoxic and oxidative effects of anatase TiO_2NPs and its suitability to study potential NM toxicity. This study is partially financed by FP7-NANoREG project, Grant n. 310584

FLOW MOLECULAR CYTOGENETIC IMPACTS REALITY: BREEDING WHEAT BY CHROMOSOME ENGINEERING AND FISHIS FLOW SORTING

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key words: *Thinopyrum ponticum, Triticum durum, Secale cereale, cytogenetics, climate and environmental changes*

The *Poaceae* family encompasses important crops, such as wheat, rice and mais, all of them staple food for humans and animals nutrition. We have dealt with wheat, for which novel breeding goals are emerging, also in the light of current and expected climate and environmental changes. Many agronomically valuable genes have been transferred into cultivated wheats by interspecific hybridization with wild relatives belonging to the *Triticeae* tribe. A deeper knowledge of sequence and function of genes of interest is highly desirable, as it can facilitate targeted breeding. This is now feasible, thanks to the fast "omics" evolution, with ever cheaper genome sequencing being one of the several open avenues available to today's scientists. However, the huge genome (12Gb) and polyploid nature of pasta wheat (4n) and of some of its wild relatives make genome analysis quite challenging. Among the several approaches to reduce genome complexity, flow cytometry and chromosome sorting have been demonstrated to be the most effective. However, standard flow cytometry analysis enables sorting of few chromosome types, otherwise peculiar cytogenetic stock are required. The development of the Fluorescence *In Situ* Hybridization In Suspension methods (FISHIS) widens the possibility to sort specific chromosomes even from chromosome engineered varieties or breeding lines of agronomic interest and marks the birth of the flow molecular cytogenetic. Here we describe the cytogenetic and flow molecular cytogenetic analysis of two interspecific breeding lines: a durum wheat/*Thinopyrum ponticum* recombinant line, containing a 7AL/7el$_1$L recombinant chromosome with several genes/QTL of proved validity, and a common wheat/rye addition line, including a pair of rye 1R long arms (1RL) fused with the satellite body (sat) of wheat chromosome 6B. Both alien chromosome types have been flow sorted and characterized further by post-sorting molecular marker and GISH analysis, respectively.

A FLOW CYTOMETRY BASED-SYSTEM FOR RAPID SCREENING OF *IN VITRO* MADE DNA CONTENT CHANGES IN *ORCHIDS*

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Key words: Flow cytometry, Cycle Value, colchicine, APM, LC-HRMS

Orchids hold a high economical value as ornamentals and for the production of some metabolites with antioxidant and anti-tumoral activities. Among others, the genus *Dendrobium* has been selected for manipulating ploidy levels to develop new varieties with increased ornamental value and a higher production of secondary metabolites. *Dendrobium* plants (e.g. *Dendrobium loddigesii* ssp.) with high content of secondary metabolites are normally harvested in the wild, grazing this species a lot till extinction risks. Here we present a new and fast flow cytometry approach to obtain and select *Dendrobium loddigesii* polyploids, through an early *in vitro* screening on Protocorm Like Bodies (PLBs) after antimitotic treatment. PLBs are a typical orchid vegetative reproductive organ which allow for fast propagation of tru-to-type individuals. Our approach allows the identification of the best treatment procedure to control cell cycle and for the assessment of effective conditions for polyploid recovery. Just one month after treatments and by using Cycle Value, we were able to discard about the two-third of the unchanged material (no DNA content variations) by drastically reducing the number of explants to work with, and the corresponding labour costs. Different conditions, regarding

concentrations and exposition time, were tested using the antimitotic compounds colchicine or amiprophos-methyl (APM). A high polyploids recovery, up to 80%, was obtained with both antimitotic agents, and those explants were further characterized by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS), to identify polyploid explants showing an increased levels in high-value molecules as shihunidine and hircinol.

FLOW CYTOMETRY INVESTIGATION IN NANOTOXICOLOGY

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Toxicity studies are important in biochemical and medical research, and essential prior to the commercial use of newly developed chemicals and nanomaterials. The current methodology of in vitro nanotoxicological screening however needs of multidisciplinary knowledge to assess the health risk, and flow cytometry technology (FCM) offers a powerfull screening method in nanomaterials characterization and genotoxicity assessment. The both physical and chemical investigation properties of the flow cytometry allow to evaluate a number of cellular parameters usefull for the comprehension of physiological and toxicological cell responces to nanomaterials exposition. Recently, we have applyed FCM to discriminate the different cytotoxic and genotoxic responce by the humans periferal blood mononucleated cell (PBMC) exposed to TiO2 particles of different size and crystalline forms. In this case, it has been possible evaluate the effects both in the monocytes as well as the lymphocytes subpopulations dimostrating the different responces that take place in term of cytotoxicity and genotoxicity in comparison to the different ability to uptake nanoparticles. Our study has confirmed the suitability of human PBMCs as a multicells model for in vitro nanotoxicology tests, but it has also suggested some caution has to be considered if stimulated lymphocytes have to be used for the assessment of clastogenicity.

CHARACTERIZATION OF REGULATORY PARTS FOR THE METABOLIC ENGINEERING OF E. COLI AND B. SUBTILIS: APPLICATIONS IN THE CONVERSION OF BIO-WASTE INTO BIO-FUELS AND BIO-PRODUCTS

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We aim to metabolically optimize two different chassis, Escherichia coli and Bacillus subtilis, respectively for the bioconversion of lactose-rich dairy waste into ethanol, and of glycerol-rich biodiesel industry waste into poly-gamma-glutamic acid (gamma-PGA). The former is a popular biofuel, while the latter is a biopolymer commonly found in fermented foods like natto, with a range of applications, including medicine and bioremediation. A typical synthetic biology pipeline was followed for the rational engineering of the two microbes, including new parts, models and genetic tools.
A new synthetic ethanologenic operon was engineered in E. coli, including heterologous pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adhB) from Zymomonas mobilis. To overcome the previously observed high transcriptional demand in engineered ethanologenic strains, genes were codon-optimized for expression in E. coli; RBSs and promoters were selected to maximize translation and find optimal expression level. Competing pathways, already identified in literature, were blocked via sRNA-based silencing or a new BioBrick-compatible allelic replacement vector (pBBKnock).
In B. subtilis, an endogenous operon (pgs, encoding a trans-membrane protein complex), normally non-expressed in laboratory strains, is responsible for gamma-PGA production. A genome-scale metabolic model of this microbe was selected, tested and adopted to find candidate deletion/over-expression targets to improve gamma-PGA production flux in vivo. The expression of the pgs operon and the identified over-expression targets was tuned to optimize product yield by exploiting a recently described quantitatively characterized promoter library.
Population and single-cell measurements were carried out in vivo via fluorescence assays on reporter genes in microplate reader and flow cytometer, and provided accurate and multi-faceted characterization of regulatory parts activity and quality. All the synthetic pathways realized in this work relied on pre-characterized toolkits to parts including high diversity and low inter-individual variability. Trial-and-error approaches were necessary to accomplish hard-to-predict tasks, such as the initial strain selection, according to fast growth/substrate consumption in waste stream. Nonetheless, the design, build and test of the two described engineered strains, greatly benefit from the followed synthetic biology approach, which significantly reduces random optimization steps.

SHIFTING OF A MICROALGAE-BACTERIA CONSORTIUM DURING THE TREATMENT OF REAL MUNICIPAL WASTEWATER IN A PHOTOBIOREACTOR

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Global warming, high energy consumption and impact of excess sludge disposal impose a paradigm shift in wastewater treatments plants (WWTP). A particularly attractive alternative is the use of microalgae-bacteria consortia in photobioreactors, where symbiotic relations between microalgae and bacteria might be exploited. Most studies in the literature combine the use of pure microalgal strains with synthetic wastewater to prevent the contamination of microorganisms (mostly bacteria) spontaneously present in wastewater. Unfortunately, this approach limits the reproduction of the real conditions in WWTPs. In addition, a scarce knowledge exists about the composition of the microbial consortia involved in photobioreactors for the treatment of real wastewater. The aim of this research was to monitor the evolution of a

suspended-biomass in a photo-sequencing batch reactors (PSBR) which treated the wastewater collected from the primary settler of the Trento Nord WWTP. The lab-scale PSBR consisted of a 1.5 L glass reactor characterized by a photoperiod of 24 h. The PSBR was initially inoculated with *Chlorella vulgaris*, but after two months of feeding with real wastewater, a shifting of the microbial community was observed. Monitoring was performed through periodical sedimentation tests, microscopic observations and flow cytometric analyses. The biomass underwent sonication (specific energy of 80 kJ/L) to disaggregate the flocs and then the pre-treated samples were stained with Sybr-Green I to produce green fluorescence (to mark bacteria), well distinguished by the autofluorescence of photosynthetic pigments (dark red in microalgae, orange/red in cyanobacteria). Forward angle light scattering was also exploited to distinguish the populations of the microalgae-bacteria consortium. The initial population of Chlorella was gradually replaced by a large variety of microalgae, cyanobacteria and heterotrophic bacteria, well distinguished with flow cytometry. Flow cytometry was also applied to quantify microorganisms in the treated wastewater effluent from the reactor in order to evaluate the amount and the type of biomass leaving the system. The application of conventional flow cytometry was limited by the presence of filamentous microalgae in the system. High removal efficiency of organic matter, nitrogen compounds, solids and microorganisms were observed in the PSBR and the effluent wastewater met the requirements for the discharge in surface water bodies.

THE USE OF FLOW CYTOMETRY TO CHARACTERIZE NUCLEAR GENOME OF AGROPYRON CRISTATUM, A WILD RELATIVE OF WHEAT

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key words: *flow cytogenetics, B chromosomes, P genome, wheat breeding*

Crested wheat grass (*Agropyron cristatum*) is a wild relative of wheat which exhibits large genetic variation and offers a pool of alleles and genes for wheat improvement. The species comprises a complex of diploid (2n=2x=14, PP), tetraploid (2n=4x=28, PPPP) and hexaploid (2n=6x=42, PPPPPP) forms. In the first part of this work, we characterized 80 accessions from different parts of the world. Flow cytometric analysis classified 26 accessions as diploid, 51 accessions as tetraploid and 3 accessions as hexaploid. These results were confirmed by chromosome counting. Mean nuclear 2C DNA content of diploid plants was estimated as 12.83 pg DNA (1C genome size = 6.27 Gbp). Some diploid genotypes exhibited slightly higher DNA content, and were identified as B-chromosome carriers. With the aim to facilitate genome mapping and sequencing, we explored a possibility of developing flow cytogenetics for the species. Flow karyotypes obtained after the analysis of DAPI-stained suspensions of mitotic metaphase chromosomes of diploid *A. cristatum* consisted of three peaks representing seven chromosomes of the species and the chromosomes could only be sorted in groups of 2 or 3. However, chromosomes 1P - 6P could be sorted individually from wheat-*A. cristatum* addition lines at purities ranging from 81 to 98 %. B chromosomes could be easily discriminated and sorted at purities higher than 95%. These results provide an attractive opportunity to investigate the structure and evolution of the P genome of *A. cristatum* and to develop molecular tools to facilitate the exploitation of this wild species and support alien introgression breeding of bread wheat. This work has been supported by the Grant Agency of the Czech Republic (award 315160-2015).

EVALUATION OF EARLY GENOTOXIC AND CYTOTOXIC EFFECTS OF OCCUPATIONAL ANTINEOPLASTIC DRUGS EXPOSURE BY BUCCAL MICRONUCLEUS CYTOME (BMCYT) ASSAY

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Currently more then 100 different antineoplastic drugs (ANDs) are used for treatment of cancer and many other pathologies. Several ANDs which inhibit tumor growth by disrupting cell division and by killing growing cells, have been evaluated by IARC that included six of them in Group 1. We aimed to evaluate early genotoxic and cytotoxic risk in nurses who administer antineoplastic drugs in oncology units of two Italian hospitals (A and B). We enrolled 17 nurses (11 in A and 6 in B) and 43 controls (28 in A and 15 in B). Workplace monitoring of 5-fluorouracil (5FU) and gemcitabine (GEM) was performed by HPLC-UV analysis on wipes and swabs collected in the areas of administering wards. Personal exposure to 5FU and GEM was monitored by pads placed on the protective clothes. Absorption of 5FU was assessed by measuring its urinary metabolite α-fluoro-β-alanine (AFBA) by LC-MS-MS. Total amount of handled drugs was also calculated. Buccal micronucleus cytome (BMCyt) assay was used to measure biomarkers of DNA damage (micronuclei MN and nuclear buds NB), cytokinetic defects (binucleated cells BN) and cell death (condensed chromatin CC, karyorrhexis, picnotic and karyolitic cells). GEM contamination was found in about 60% of wipe and swab samples. In the different areas, contamination for GEM ranged from 0.64-93.90 μg (median values 7.17 μg in A and 2.93 μg in B) and for 5FU from 0.26–196.83 μg (median values 0.90 μg in A and 3.31 μg in B). Only GEM deposition (0.18-15.21 μg) was found on workers' pads and median value was higher in A than in B (5.06 vs 0.30 μg), with total amount of 115.26 μg in A and 4.20 μg in B. No AFBA was found in urine samples. Total amount of administered drugs was higher in hospital A (710g) than in B (124g). In A we found in nurses also the highest frequencies of MN (4.3‰) and CC (6.7‰) which were significantly higher than respective controls (1.5 and 2.7‰). Also in B, nurses showed higher frequencies of MN, CC and MN+Nuclear buds (3.5, 2.7 and 7.6‰ respectively) than their controls (1.1, 1.0 and 2.1‰ respectively). In conclusion, in both the hospitals, the nurses who administer ANDs, showed early genotoxic and cytotoxic effects suggesting the need to be more careful during AND administration. BMCyt assay seems to be an interesting biomarker of early cytotoxic and genotoxic effect for occupational antineoplastics exposure because it is sensitive

and no invasive, although it needs to be tested on a higher number of subjects.

RESOLVING SINGLE CHROMOSOMES OUT OF THE BUSH: IMAGING FLOW CYTOMETRY GIVES MORE POWER TO FLOW MOLECULAR CYTOGENETICS

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key words: FISHIS, chromosome and nuclei suspension, wheat, oat, lymphocyte

Imaging flow cytometry (IFC) combines the qualitative imaging analysis capabilities of microscopy with the high throughput and statistical capabilities of flow cytometry. Technical advances in optics and electronics have encreased instrument resolution and speed analysis on cells and chromosomes in suspension making feasible data collection from thousands of elements in a short time. For the first time, we have applied IFC to FISHIS samples of plant chromosomes and nuclei revealing the power of this technique to characterize undistinguishable chromosomes and nuclei morphology in oat, wheat and human limphocytes too. By using the 60x magnification, with the EDF option enabled (high focus deep), resolution was sufficient to discriminate single chromosomes in suspension and tracking their position and numerosity in the sample. We envisage that this high-throughput analytical approach has a major relevant feature in respect to flow cytometry alone, which is related to evaluate and "visualize" the hybridization marks. FISHIS signals can then be analysed for intensity, position and number, joining the quantitative approach of FCM to the visual interpretation of the spots topology, thus facilitating mutation scoring and diagnosis. Furthermore, FISHIS beeing a faster and simpler procedure in respect to standard FISH or S-FISH, could facilitate and extend the use of cytogenetics for analitical and clinical purposes both in plant biotecnologies (chromosome characterization for a driven sorting) and cancer diagnoses (e.g. mutations on lymphocytes chromosomes during leukemia desease).

Hematology

HIGH-THROUGHPUT IMMUNOPHENOTYPIC CHARACTERIZATION OF BONE MARROW- AND CORD BLOODDERIVED MESENCHYMAL STROMAL CELLS REVEALS COMMON AND DIFFERENTIALLY EXPRESSED MARKERS. RELEVANCE OF ANGIOTENSIN-CONVERTING ENZYME (CD143)

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Background: Mesenchymal stromal cells (MSC) are a heterogeneous population of multipotent progenitors used in clinic for their regenerative and immunomodulatory properties. Although bone marrow (BM) remains the most common MSC source, cord blood (CB) is an alternative, which can be collected non-invasively and without major ethical concerns. To the best of our knowledge, comparative studies comprehensively characterizing MSC phenotype across several tissue sources are still lacking. The present study provides a 246 antigens immunophenotypic analysis of BM- and CB-derived MSC, aimed at identifying common and strongly expressed MSC markers as well as the existence of discriminating markers between the two sources.
Results: Based on unsupervised hierarchical cluster analysis we have identified four groups of markers according to their high to negative expression. To find the most common MSC markers regardless of the source, we focused on the highest expressed antigens: alpha-SMA, beta-2-microglobulin, CD105, CD13, CD140b, CD147, CD151, CD276, CD29, CD44, CD47, CD59, CD73, CD81, CD90, CD98, HLA-ABC and vimentin. All excepting CD140b and alpha-SMA were suitable for the specific identification of ex-vivo expanded MSC. Conversely we found that only one out 246 markers, angiotensin-converting enzyme (CD143), was exclusively expressed by BM-MSC. CD143 expression was further validated on 10 additional BM- and CB-MSC samples and on 10 umbilical cord- and adipose tissue- derived MSC confirming that its expression was restricted to adult sources.
Conclusions: We have identified specific markers shared and strongly expressed by BM- and CB derived MSC that could complement the minimal panel proposed for in-vitro MSC definition. We have also identified CD143 as a marker exclusively expressed on MSC derived from adult tissue sources. Further studies are needed to elucidate the biological role of CD143 and its potential association with tissue-specific MSC features.

CIRCULATING CD172A^{+} HUMAN CARDIAC MICROVESICLES ARE A NEW BIOMARKER OF MYOCARDIAL ISCHEMIC STRESS

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The discovery of microvesicles (MVs) has improved our knowledge of intercellular communication. The presence of MVs in various body fluids suggests their potential as biomarkers. Indeed, it is still felt the need of a method for selectively characterize and quantify circulating tissue-specific MVs, in particular cardiac-derived microvesicles. Here, we describe a flow cytometry-based method for the characterization of cardiomyocyte (CM)-derived MVs, using a surface antigen CD172a, expressed in human CMs. Noteworthy, $CD172a^+$ cMVs contained the cardiac-specific troponin T and two cardiac-enriched microRNAs, miR-1 and miR-133. Myocardial stress was found to be a major determinant of cMV release *in vivo*, as assessed by plasma measurement in patients with different types of myocardial disease while hypoxia induced cMV release from human (h) CMs *in vitro*. Additionally, we demonstrated for the first time a functional role of cardiac MVs in promoting cardiomyocyte (CM) inotropism in vitro as well as the presence of ceramide-enriched lipid platforms on cardiac MV surface as a potential molecular candidate in mediating MV function under ischemic stress conditions.
Finally, aortic stenosis patients undergoing transcatheter aortic valve replacement with elevated plasma cMVs showed a more favourable prognosis compared to those with lower plasma levels.
In summary, we defined a method for isolating and measuring cMVs, demonstrated the MV release mechanism and the prognostic potential in myocardial disease.

TYPICAL FLOW CYTOMETRY PANEL IN TREATMENT-NAÏVE PATIENTS AFFECTED BY CHRONIC LYMPHOCYTIC LEUKEMIA WITH TRISOMY 12

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Introduction: Patients (pts) affected by chronic lymphocytic leukemia (CLL) and FISH positive for trisomy 12 (+12) have unique clinical and biological features. We, therefore, performed a literature review on the known association between demographic, clinical, laboratoristic and biological features in treatment-naive pts with +12 CLL and we focused on characterising the flow cytometry panel of these pts.
Methods: The study included 487 treatment-naive pts with +12 CLL from 16 centers. These pts, diagnosed between January 2000 and July 2016, were compared to a control group of 816 treatment-naive pts with FISH negative CLL, matched by age and gender and followed in the same centers. An additional cohort of 250 pts followed at a single US institution was used as external validation.
Results: The collection of data regarding pts with +12 showed positivity for ZAP70 (≥20%) in 54.3% of the pts (197 out of 363 available data), CD38 (≥30%) in 51.3% (222 out of 433) and CD49d (≥30%) in 78.8% (89 out of 113). The data regarding pts with negative FISH were the following: positivity for ZAP70 in 35.6% (241 out of 676), CD38 in 27.7% (213 out of 768) and CD49d in 26.9% (54 out of 201). As compared to pts with negative FISH, pts with +12 had a significant higher prevalence of ZAP70 positivity ($p<0.001$), CD38 ($p<0.001$) and CD49d ($p<0.001$).
These data were confirmed also when using the external validation group of +12 pts for ZAP70 positivity ($p=0.03$) and CD38 positivity ($p=0.004$); data on CD49d were not available.
The preliminary study conducted on a single-centre cohort (60 pts with +12 vs. 60 pts with negative FISH) allowed us to perform a more specific analysis including also CD69 positivity and data on expression percentage and Mean Fluorescence Intensity (MFI) of ZAP70, CD38, CD49d, CD20 and CD11c.
The comparison between the 2 groups showed a significant difference for ZAP70 positivity ($p=0.002$), CD38 positivity ($p<0.001$), CD38% ($p<0.001$), CD38 MFI ($p<0.001$), CD49d positivity ($p<0.001$), CD49d% ($p<0.001$), CD49d MFI ($p<0.001$), CD20% ($p=0.017$), CD20 MFI (<0.001), CD11c% ($p=0.048$) and CD11c MFI ($p=0.002$). No significance has been found for CD69 positivity.
Conclusions: Our study on 487 pts with +12 CLL and the analysis on 250 pts of the validation cohort showed that pts with +12 have a typical flow cytometry panel characterized by ZAP70 and CD38 positivity; smaller cohorts of pts showed also distinguishing positivity for CD20 and CD11c.

FLOW-CYTOMETRIC DIAGNOSIS OF SMALL CELL LUNG CANCER: TWO CASE REPORTS IN A SINGLE CENTER EXPERIENCE

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We describe two cases of immunophenotypic diagnosis of small cell lung cancer (SCLC) in two female patients.
In particular, a 73-yo woman presented severe dyspnea, pallor and hepatomegaly. Laboratory studies showed anemia (Hb, 96 g/L), leukocytosis (WBC, $13.62x10^9$/L) with neutrophilia and thrombocytopenia (PLTs $25x10^9$/L), elevated LDH, total bilirubin 2.4 mg/dL. PB smears documented the presence of erythroblasts (8/100 WBC) and immature granulocytes (13%). The BM aspiration showed an acute leukemia morphology with hypercellularity and 90% of blast cells, sometimes vacuolated, with basophi-

lic cytoplasm and negative to myeloperoxidase (MPO). The flow cytometric analysis on BM aspirate revealed the presence of a small and low complex population with a negative CD45 and a bright expression of CD117 and CD56. This population resulted negative for CD34, CD13, CD33, HLA-DR, MPO, CD36, CD19, CD38, CD7, CD3 (surface and intracytoplasmic), CD71, CD105. The observed immunophenotype did not match any WHO classification of hematological malignancies. BM biopsy confirmed the neoplastic involvement by SCLC cells.
The second case was a 74-yo female patient admitted to the Geriatric department of our hospital with the clinical suspicion of pneumonia. The CT scan documented the presence of a mediastinal mass of 9,8X8,4X9,4 cm with right lung parenchima involvement. A transesophageal biopsy was performed. In our laboratory the cell suspension was prepared by mechanical disaggregation of the solid tissue using the Medimachine system. Because of the clinical suspicion of a lymphoproliferative disorder, the screening panel included CD19, CD20, CD10, Kappa, Lambda, CD5, CD3, CD4 and CD8. Nevertheless, we observed a physiological B-cell population with a normal kappa/lambda ratio and no aberrant T cell pattern. A CD45 negative population with the same immunophenotypic pattern of the previous case was observed. Immunohistochemistry confirmed the extraematological origin of this population.
SCLC is a neuroendocrine carcinoma with aggressive behavior with early spread to distant sites.
In these two cases flow-cytometric studies showed a specific antigenic pattern for diagnosis of SCLC (CD45 neg, CD117 pos, CD56 pos). In this context, flow cytometry on BM aspirate and/or ago-biopsy is able to guide towards an appropriate diagnosis of an unknown tumor, despite a clinical suspicion of an hematological neoplasm.

IPLASMA CELLS AND PLASMA CELL TUMORS ARE THE LONE MET EXPRESSORS AMONG HEMATOPOIETIC SYSTEM

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Background. The MET oncogene was reported to be expressed by normal plasma cells and myelomatous cells. Furthermore, in acute myeloid leukemia (AML), MET activation was shown to sustain proliferation and survival. Here we investigated MET expression in a panel of hematological malignancies.
Methods. Bone marrow (15 normal, 9 AML, 13 B cell chronic lymphoproliferative diseases - BLPD), peripheral blood (7 AML, 28 BLPD) and tissue samples (11 BLPD) were analyzed for MET expression by flow cytometry using the following mixture of monoclonal antibodies: CD38, a-MET, CD20, CD34, CD33, CD10, CD19, CD45. Gating strategy on normal bone marrow allowed analysis of MET on lymphocytes (B and T+NK, Plasma cells, B cell precursors), CD34+ myeloid precursors, granulocytopoietic population and erythroid cells. Similarly, on pathologic samples, neoplastic population was gated on to evaluate MET expression.
Results. Met expression was detectable only in one case of Multiple Myeloma and one case of Diffuse Large B Cell Lymphoma with plasmablastic differentiation, i.e. the only two cases in our series that displayed plasma cellular differentiation. Moreover, MET was detectable in a variable percentage of plasma cells from normal bone marrows. In all other hematological malignancies analyzed, MET was undetectable either in the neoplastic populations or in normal blood and bone marrow cells. In the two positive cases, MET expression was specifically associated with $CD38^{bright}CD45^{low}$ neg plasma cells.
Conclusions. MET expression is associated with the advanced stages of normal and malignant B-cell differentiation, but it is never observed in any other blood cell subpopulation, either neoplastic or normal, including mature and stem/progenitor cells. The functional role of MET in plasma cells is currently unknown, but, according to the anti-apoptotic properties of this receptor, could promote resistance to chemotherapy.

CLINICAL FLOW CYTOMETRY REPORT: A PRELIMINARY CONSENSUS STATEMET

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Background: The Italian Cytometry Sociaty (GIC) in collaboration with the GPMI (Gruppo Policentrico Marcatori Immunologici) has supported among the activities for professional accreditation in flow cytometry a preliminary project for the standardization of clinical flow cytometry reporting.
Aim: To standardize reporting criteria among different flow cytometry laboratories and to obtain a shared standard core template for flow cytometry reporting.
Methods: 41 professionals, belonging to 34 different flow cytometry laboratories recruited from all over the national territory, agreed to join the project. In order to estimate the level of agreement on the core aspects and minimal requirements of the clinical flow cytometry report a questionnaire was prepared. A total of 27 questions, composed by 17 main queries some divided in sub questions, were devised. Four main topics were identified: 1) patient's general information (personal data and patient identification); 2) request specificities (sample characteristics, qualitative and quantitative analysis results); 3) criteria on how to draw the final interpretations; 4) responsibility. Any "YES" answer was given a score of 1 point, while any answer "DIFFERENT THAN YES" was given 0 points. The degree of agreement was measured by calculating the % of "YES" out of the total answers received.
Results: in 13/27 questions an agreement of ≥75% was found; in 8/27 the agreement was ≥90%; in 8/27 the agreement was between 61% and 75%; in 5/27 was between 40% and 61%.
Conclusions: The results of this project suggest an initial

consensus on what should compose the core template of a clinical flow cytometry immunophenotyping report, although some aspects are still under debate and need further discussion. The proposals emerged from this query should therefore be considered as only preliminary and only in regards of the core template of the clinical report.

CYTOFLUORIMETRIC APPROACHES TO STUDY *NOTCH1* MUTATIONS IN CLL WITH *IN VITRO* AND *IN VIVO* STRATEGIES

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Since their identification, *NOTCH1* mutations in CLL were associated with unfavorable outcome, high risk of disease progression and drug resistance. These mutations are already found at earlier stages of the disease, mostly at the subclonal level, often increasing at the time of progression. However, the mechanisms driving clonal evolution remain still unclear. In order to investigate if deregulated *NOTCH1* pathway activation can confer a proliferative and/or anti-apoptotic advantage to the mutated clone, contributing to a progressive selection over the wild-type (WT) one, we exploited our CRISPR/Cas9-generated Mec-1 model (Mec-1/WT and /PEST). To univocally identify the different Mec-1 variants both *in vitro* and *in vivo*, cells were transduced to express eGFP (maximum excitation and emission at 488 and 509 nm, respectively) or iRFP670 (maximum excitation and emission at 643 and 670 nm, respectively). These clones were mixed in a 1:1 proportion and cultured over a layer of stromal cells (HS-5) overexpressing NOTCH1 ligands, either DLL1 or Jagged1. Results obtained from FACS analysis of mixed populations indicated that PEST-mutated cells grow significantly faster than WT ones in the presence of stromal cells, becoming the dominant population within a 7-day co-culture and suggesting that microenvironment-dependent activation of NOTCH1 pathway plays a determinant role in driving mutated cells. Furthermore, after the co-culture period, the two clones were sorted (taking advantage of their fluorescence and of a CD19-PECy7 counterstaining) to comparatively assess their gene expression profile, both at the baseline or after stromal cell contact. Mixed populations of fluorescent cells were also used *in vivo*: NSG mice were intravenously injected with fluorescently labeled WT and PEST clones either as mixed or as single cells population and left to engraft. Disease spreading was monitored weekly by FACS analysis of explanted organs, and results indicated a predominant spleen involvement determined by PEST cells. This suggests that, in a heterogeneous leukemic population, *NOTCH1*-mutated cells are more prone than WT cells to reach privileged lymphoid niches that can sustain their survival. Taken together, our data highlight a bilateral interplay between leukemic cells and the microenvironment show that PEST-mutated cells are strongly influenced by the microenvironment that favors their expansion over the WT ones by impacting on growth and migratory ability.

BAL: A LIQUID LUNG BIOPSY

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Cells and non-cellular components present in the bronchoalveolar lavage fluid are representative of the inflammatory and immune system of the distal airways. The analysis of the immunephenotype of cells present in the BAL allows classification, determination of vitality, maturation stage and clonality. Using this technique, we can study lymphocytes, histiocytes, mast cells, eosinophils, neutrophil granulocytes and macrophage population. Important things to do for a simple bronchial lavage to become lung biopsy are a satisfactory anamnestic exchange with the clinician and an adequate antibody panel to use. What antibodies to use? In this Flow Cytometry Unit we added CD36, CD15 and CD14 for macrophage study, CD117 and HLA-DR for mast cells, antibodies versus κ and λ light chains, CD13, CD16 and CD9 for eosinophils, in addition to the basic antibodies for the study of lymphocytic populations (CD3, CD19, CD16, CD56, CD8, CD4, HLA-DR) and granulocyte population (CD16, CD11b). The study of the lymphocytic component allows us to discriminate between lymphoproliferative processes and lung interstitial pathologies and within these we can make fine differential diagnosis. The only evaluation of the percentages of the various cell populations present in the BAL allows us a first diagnostic orientation: a lymphocyte percentage greater than 15% is mainly associated with sarcoidosis and pneumonia caused by hypersensitivity to radiation or induced by drugs; a lymphocytosis > 25% is associated with lung granulomatous pathologies or lymphomas; a lymphocytosis > 50% is associated with pneumonia caused by hypersensitivity. As regards the myeloid population, a granulocyte component > 3% may be a sign of fibrosis or lung infection; a neutrophilism > 50% may be a sign of lung suppurative infection; a eosinophils percentage >1% can mean asthma or allergic bronchopneumonia, if greater than 25% acute eosinophilic pneumonia. These diagnostic potentialities allow us to consider BAL a liquid biopsy of lung, a complement to the high-resolution TAC, and contributed to the reduction of pulmonary biopsies. More diagnostic novelties will come from the cytometric study of functionality and cytokines release of BAL lymphocytes.

FLOW-CYTOMETRIC ASSESSMENT OF LYMPH NODE SUSPENSIONS WITH CLINCAL SUSPICION OF NON-HODGKIN LYMPHOMA. A SINGLE CENTER EXPERIENCE.

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BACKGROUND. Few studies have addressed the utility of flow cytometry on tissue samples of lymph nodes. A high concordance between results of flow cytometry and immunohistochemistry has been reported in a single large series of patients with non-Hodgkin lymphoma (NHL).
AIM. We report our experience with a flow-cytometric diagnostic approach to lymph node tissue in patients with a clinical suspicion of lymphoproliferative disorders.
METHODS. Lymph node suspensions were prepared by mechanical disaggregation of the of solid tissue using the

Medimachine system (Becton Dickinson). The screening panel included CD19, CD20, CD10, Kappa, Lambda, CD5, CD3, CD4 and CD8. Further monoclonal antibodies were used in selected cases. Results of immunophenotyping were compared with immunohistochemical diagnoses.

RESULTS. We assessed 53 lymph node suspensions by flow cytometry. Immunohisotchemical diagnoses were diffuse large B-cell lymphoma (DLBCL) in 16 patients, follicular lymphoma (FL) in 7 patients, mantle cell lymphoma (MCL) in 1 patient, marginal zone lymphoma (MZL) in 1 patient, Hodgkin lymphoma (HL) in 12 patients, T-cell lymphoblastic lymphoma (T-ALL) in 1 patient, mycosis fungoides (MF) in 1 patient, anaplastic large-cell lymphoma (ALCL) in 1 patient, metastatic carcinoma in 5 patients, reactive lymph node changes of various types in 8 patients. All B-subtype non-Hodgkin lymphomas (B-NHL) were correctly identified (100% sensitivity) by flow-cytometric analysis. B-cell pathological populations were CD19 and CD20 positive and presented a clear clonal light chain restriction in 18 cases out of 25, while 7 cases did not show any light chain expression. CD10 was expressed in all FL cases and in 3 out of 16 DLBCL (19%). CD5 was present in the patient with MCL and in 1 case of DLBCL. In non B-NHL samples, we observed a physiological B-cell population with a normal kappa/lambda ratio (100% specificity). Lymph node suspension of MF presented the typical phenotype CD4+/CD26-/CD7±. ALCL patient showed a pathological population CD45+/CD30+bright/CD2+.

CONCLUSIONS. In our limited series of patients, flow-cytometric assessment of lymph node suspensions had a high sensitivity and specificity for NHL. It could be useful to provide the clinician and pathologist with rapid information to guide further diagnostic work-up and direct therapy in symptomatic patients with bulky disease.

SURFACE ENDOGLIN (CD105) EXPRESSION ON ACUTE LEUKEMIA BLAST CELLS: AN EXTENSIVE FLOW CYTOMETRY STUDY OF 1,002 PATIENTS.

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Endoglin, a receptor of the transforming growth factor-beta superfamily, is considered a powerful marker of angiogenesis and a potential main player in the pathogenesis of vascular diseases and in tumor progression. Just a little has been done as concerns endoglin/CD105 expression on hematopoietic neoplastic cells in large series of blood malignancies.

The substantial lack of cytometric data on malignant hematopoiesis prompted us to analyze by flow cytometry the expression of CD105 in a series of 1,002 de novo acute leukemias, using the PE-conjugated anti-CD105 monoclonal antibody (R&D Systems, MN USA). According to the comparison between CD105 staining and internal negative controls, single cases were classified as CD105-positive or CD105-negative. A CD45 vs side scatter or vs transferrin receptor/CD71 dot-plot was used to distinguish erythroid from non-erythroid cells.

Overall, endoglin was expressed on blast cells in 246/666 (or 36.9%) cases of acute myeloid leukemia (AML) and in 230/336 (or 68.4%) of acute lymphoblastic leukemia (ALL). Among "Not Otherwise Specified (NOS) AML", those with minimal differentiation expressed the antigen in 27/40 cases (67.5%), AML without maturation in 16/46 cases (34.7%), AML with maturation in 73/156 cases (46.2%), acute myelo-monocytic leukemia in 51/187 cases (27.2%), acute monoblastic/monocytic leukemia in 12/37 cases (32.4%), pure erythroid leukemia in 3/6 cases (50%), acute megakaryoblastic leukemia in 3/4 cases (75%). The difference between the first three types and myelo-monocytic plus monoblastic/monocytic leukemias was statistically significant ($p<0.0001$).

Patients showing the t(8;21) translocation, displaying the AML1-ETO hybrid gene, expressed the molecule in a high fraction of cases (32/41 cases, or 78%). In spite of similar pathogenesis, inv(16) AML, as compared to t(8;21) AML, expressed CD105 in a lower percentage of cases (6/24, or 25%, $p <0.01$). The absence of CD105 in 100% of APL (0/54 cases) can be considered a new relevant phenotypic marker of this leukaemia subtype. We also found that CD105 was prevalently expressed in 11q23 AML cases (11/35 or 31.4%) as compared to $NPM1_{mut}$ AML (2/19 or 10.5%). Finally, 10/17 cases of AML with biallelic mutations of CEBPA (58,8%) were found to express CD105, a percentage significantly higher than in other AML with recurrent genetic abnormalities, with the exception of those with t(8;21).

CD105 was expressed in the majority of cases of acute lymphoblastic leukemia (ALL) of B-cell origin (B-ALL). Analyzing the various B-ALL subsets, only the most immature types expressed the antigen (226/276 cases or 81.8%). Interestingly, CD105 was absent in "Burkitt-like" B-ALL with mature phenotype (0/13 cases).

In ALL of T-cell origin (T-ALL) CD105 was, again, associated to undifferentiated cases. In fact, taken together, T-ALL expressed CD105 in 8.5% of patients (4/47 cases). Early-T leukemia, i.e. the most immature type, expressed the antigen in 25% of cases (3/12), while more differentiated T-lymphoblastic leukemia/lymphomas (T-LL) showed CD105 in 2.8% of cases (1/35, $p=0.04$).

In conclusion, we report here an expression pattern never described before, in which CD105 molecule is able to identify the most undifferentiated stages of neoplastic cell differentiation in acute leukemias. Overall, the data produced in this study suggest a potential role of anti-CD105 TRC105 monoclonal antibody in almost half of all acute leukemia cases.

STEM CELLS MOBILIZATION IN SEPTIC SHOCK SURGICAL PATIENTS

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Introduction: Septic shock is the leading cause of death wordlwide. Several efforts have been made to study the stem cells role in septic patients, but we currently lack of informations regarding septic surgical patients.

Objectives: This study aims to evaluate the time course

level circulating CD34/CD133 stem cells in septic shock patients undergoing major abdominal surgery.
Methods: In the prospective observational study were enrolled consecutive patients undergoing major abdominal surgery at University Hospital, Foggia. After written informed consent, blood samples were collected on admission of the post surgical patients in intensive care unit (ICU) or in surgery ward at 24 hours (T1), and 3 (T3), and 7 (T7) days postoperatively. Quantitative analysis of endothelial progenitor stem cells (CD34) was performed. At any time were also collected the clinical parameters, the laboratory values of interleukins, procalcitonin, C-reactive protein and endotoxin, white blood cells, red blood cells, hemoglobin and platelets. The outcome was also recorded. The data are presented as median±SE.
Results: 36 patients undergoing major abdominal surgery were analysed. 21 patients with septic shock required ICU admission following surgery and 15 patients (controls) were admitted in post-surgical ward. 15 of 21 patients with septic shock deceased within 15 days postoperatively. At T1, CD34 cells were 0.23±1.5/mcl in control group, 0.06±0.1/mcl in septic shock survivors and 0.32±2.2/mcl in septic shock non survivors (NS). At T3, the circulating stem cells slightly increased in septic shock survivors and control while they reduced, whereas non significantly, in non survivors patients (0.2±1/mcl, 0.27±1/mcl, 0.16±2.5 respectively). At T7 the CD34 stem cells were slightly higher in septic shock survivors as compared to control and non survivors (0.36±0.1/mcl, 0.21±0.06/mcl and 0.25±0.4/mcl, respectively).
Conclusions: The preliminary data show increased level of the circulating CD34 stem cells in the blood of surgical patients likely due to an adaptive response to surgical stress. Moreover it appears that patients with increased mobilization of CD34 stem cells exhibit a better outcome following major abdominal surgery. This pilot study encourages the continuation of the research project on a larger sample size of patients to better understand if and how CD34 stem cells are involved in the surgical patients with septic shock.
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CD200 EXPRESSION IN B-CELL CHRONIC LYMPHOPROLIFERATIVE DISORDERS IN LEUKEMIC PHASE.

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Despite morphology is still considered as the principal basis for the identification of lymphoid neoplasms, sometimes, however, it fails to discriminate among several B-cell chronic lymphoproliferative disorders (B-CLD). Improved immunophenotyping has resulted in a better characterization of a number of variant of these diseases, some of which may benefit from different therapeutic approaches. In 1990th the Royal Marsden British Group in London (Matutes et al, 1994; Moreau et al, 1997) proposed a score system based on 5 markers (CD5, CD23, CD79b, FMC7, SmIg) each one of them receiving a score of 1 if present and 0 if absent. Chronic lymphocytic leukemia (CLL), the most frequent form of B-CLD typically scored 4-5, while low grade lymphomas in leukemic phase scores 0-1. However, some overlap in diagnosing these forms still exist, to date. More recently, CD200 has been described as a marker potentially useful to distinguish CLL from mantle cell lymphoma (MCL).
A large cohort of patients with B-CLD (207 patients with B-CLD; M 131, 63%; F 76, 37%; mean age 67 years; range 35-88 years) consecutively seen at our Institutions were analyzed aiming at evaluate whether the addition of CD200 to the Matutes score improves our diagnostic accuracy. Of them 149 (72%) were diagnosed, based on investigator assessment, as CLL and 58 (28%) as non-CLL. These latter were: 27 marginal zone lymphoma (MZL); 13 Hairy cell leukemia (HCL); 11 follicular lymphoma (FL); 6 MCL; 1 prolymphocytic leukemia (PL).
McNemar’s test was used to assess the accuracy of the single marker assessment to determine the diagnosis of CLL. Among the analysed markers, CD23 was the only one without a statistically significant difference between the investigator assessment and the cytofluorimetric analysis. Matutes score was calculated including CD5, FMC7, CD79b, CD23 and SmIg. A modified Matutes score was calculated assessing the standard Matutes score adding one point for CD200 positivity. Sensitivity of scores for the diagnosis of CLL (based on investigator assessment) were as follows: Matutes score ≥3: 92%; Matutes score ≥4: 63%; modified Matutes score ≥4: 92%; modified Matutes score ≥5: 63%. Specificity of scores were: Matutes score ≥3: 100%; Matutes score ≥4: 100%; modified Matutes score ≥4: 100%; modified Matutes score ≥5: 100%.
These results, despite confirming the usefulness of CD200 in differential diagnosis of B-CLD, show that in our hands the inclusion of CD200 in the Matutes score does not improve its diagnostic accuracy.

PROGNOSTIC RELEVANCE OF OXIDATIVE STRESS MEASUREMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA

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The oxidative stress and the antioxidant defense status of 165 untreated patients with chronic lymphocytic leukemia (CLL) were tested and correlations with clinical-biological features and prognosis were evaluated. The reactive oxygen metabolites (ROMs) and the biological antioxidant potential (BAP test) were tested at diagnosis. A significant increased oxidative damage (d-ROMs test: 432.9 U CARR ± 140.6; range 92 – 819) and a significant reduced antioxidant potential (BAP test: 2,088 µmol/L ± 402.1; range 405.6 – 4,084) were found in CLL patients with respect to normal controls (d-ROMs test: 317.2 U CARR ± 88.3; range 158 - 497; BAP test: 2,388 µmol/L ± 251.7; range 1,876 – 3,045) (p <0.0001). CLL patients with higher d-ROMS values had higher number of circulating white blood cells and lymphocytes, and higher values of β2-microglobulin. Higher d-ROMs values were found in female (p 0.0003) and in patients with unmutated IgVH status (p 0.04), unfavourable cytogenetics (p0.002), and more advanced clinical stage (p 0.002). Higher BAP test values were found in patients with CD49d positive CLL (p 0.01) and with more advanced clinical stage (p 0.004). At a mean follow-up of 48 months, CLL patients with d-ROMs ≥418 U CARR were found to have a lower time to first treatment (TFT) with respect to others (37 months vs 130 months) (p 0.0002), and a reduced survival (p 0.006). CLL patients with BAP test values ≥2,155 µmol/L had a shorter TFT (p 0.008) and a shorter survival (p 0.003). OS can affect CLL patients by concomitantly increasing ROS production (as indicated by the risen values of d-ROMs test) and decreasing antioxidant defences (reduced values of BAP test), respectively.

MONOCLONAL B-CELL LYMPHOCYTOSIS (MBL) AND PROSTATE CANCER: AN UNEXPECTED, POSSIBLE ASSOCIATION

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Background: Monoclonal B-cell lymphocytosis (MBL) is a recognized entity characterized by the presence of a peripheral monoclonal B-cell population lower than 5000/µl, in the absence of any type of clinical features. MBL clones may have: chronic lymphocytic leukemia (CLL-like), atypical CLL or non-CLL phenotype. MBL can be also distinguished in "low-count" (<500/µl) and "high-count" (>500/µl) subtypes. High-count MBL frequently shows typical CLL phenotypic/genetic features and require adequate follow-up. MBL showing a clonal B-cell count higher than 1000-1500/µl are defined "clinical" MBL. Using highly sensitive (i.e. >6 colors and >500000 events) flow cytometry approaches, CLL-like MBL clones have been found at a frequency of 7-12% in healthy subjects, showing, however, very low median counts of clonal B-cell (10-170/µl), with 0,14% being clinical MBL.
Though several studies have described the association between CLL and various types of neoplastic disorders, only few data exist about the risk of non-hematologic cancer in individuals with MBL; in particular, no association between MBL and prostate cancer (PC) has been so far reported.
Aims: To study prospectively the frequency of CLL-like MBL (CD5+, CD19+, CD23+, CD20 dim) clones in patients affected by PC compared to healthy males of the same ages.
Methods: We enrolled 34 consecutive patients affected by PC (mean age 74 years, range 58-91), naïve for chemotherapy. All patients were planned to receive whole-pelvis RT with radical (n.23) or salvage (n.11) intent. Fifty-four healthy males (mean age 71 years, range 58-87) represented the control group. Immunophenotypic analysis of peripheral lymphocytes before RT was performed by BD FacsCanto II flow cytometer, using a 5-6 colors approach and the following antibody combinations: CD19/CD5/CD45/CD20/CD23; Kappa/Lambda/CD19/CD20/CD5/CD45. For each sample, 100000 events were collected.
Results: In PC patients, we found 3 MBL (8,8%), two of which were "high count/clinical" MBL (5.8%). In contrast, in healthy subject group, only one "low count" MBL (1.8%) was detected, showing a very small clone (8 cells/µl). Such a difference was not statistically significant (p = 0.2).
Conclusion: The preliminary results of our prospective study, performed using a routine, not highly sensitive flow cytometry approach, highlight a possible association between (clinical?) MBL and PC, never described before and probably warranting further investigation in a larger number of patients.

CD26 AND CD39 EXPRESSION ON INFILTRATING CD4 CELLS IN THE LYMPHOMA TISSUE SAMPLES

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In our previous work we analyzed in lymph nodes by flow cytometry the rich infiltrated characterizing the microenvironment of Hodgkin lymphoma (HL), mainly comprised of CD4 T.We confirmed that the majority of these CD4 T expressing the activation markers (CD38) but lose the CD26 and we suggested to identify the subset $CD4^+CD26\text{-}CD38^+$ to identify the non-neoplastic cellular pattern in HL. A subset connectable to regulatory T (Treg) cells, because the low expression of CD26 (DPP4) added to the presence of CD39 (NTPDase) may be responsible for the generation of adenosine, which plays a major role in Treg-mediated immunosuppression. AIMS: We wanted to test if this subset may also characterize T lymphocytes infiltrating the lymph nodes of non-Hodgkin's lymphomas (NHL) and to verify the expressions of the two enzymatic markers (CD26, CD39) in microenvironments of HL and NHL analyzed by FC. METHODS: we analyze by FC in lymph nodes of 6 HL and in 32 NHL (12 DLBCL,10 FL,5 SLL,3 MZL,2 MCL) the CD4 T testing the expression of CD26, CD38, CD39. RESULTS: In CD4 T of HL, CD39 is expressed in 44% of the subset and the increased presence (50%)of $CD4^+CD26\text{-}CD38^+$cells is confirmed. Compared with HL,

the cells of DLBCL are not statistically (t Student test) different: CD38 (64 vs 55; p = 0,39), CD26-CD38$^+$ (50 vs 46; p=0,66), CD39 (44 vs 59; p=0,15). While HL and FL cells are significantly different:CD38 (64 vs 23; p< 0,05), CD26-CD38$^+$(50 vs 18; p< 0,05), CD39 (44 vs 23; p<0,05).The other three types of NHL, few in number, show a tendency to a significant difference compared with DLBCL.CONCLUSIONS: Our data support the phenotypic variations in the microenvironment of different types of lymphoma emphasizing DLBCL similarity with HL and difference with FL and other NHL.They also suggest a relationship between activated environment (CD38$^+$) and high CD39 level, which, in addition to a low CD26,could enhance the generation of adenosine and, therefore, increased immune suppressive activity.The profile of CD4 T infiltrating cells by FC might be useful to characterize lymphomas environment indicating also signals and biological mechanisms representative of possible therapeutic target

CLINICAL AND LABORATORY PARAMETERS PREDICT RITUXIMAB-INDUCED ADVERSE REACTIONS THAT ARE CD20 EXPRESSION INDEPENDENT

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During the last years Rituximab has emerged as an effective therapy for CD20+ B-cell malignancies and autoimmune disorders. Despite usually well tolerated, Rituximab infusion, however, either alone or in combination with other agents, may be followed by adverse drug reactions (ADR) that may cause its discontinuation. Here we report the final data of a multicentre retrospective study aiming to evaluate real incidence, types and possibility to predict ADRs in Rituximab-treated patients.
A total of 2,846 Rituximab infusions given to 374 patients were analyzed. Overall, infusion-associated ADRs occurred in 88 (23.5%) patients, more frequently in indolent non-Hodgkin lymphomas (NHL) (38.6%), followed by chronic lymphocytic leukemia (CLL) (26%) and high-grade NHL (18%). Eight percent of patients with hemic or other types of autoimmune disorders developed ADRs, usually mild to moderate. In 8 cases (2.1%) a grade 3-4 toxicity was observed and in 4 of them (1% of all patients) definitive drug discontinuation was necessary.
Overall, the occurrence of ADR was not relevant for the quality of response to therapy (p=0.16), was not related to simultaneous or deferred infusion of Rituximab after the first cycle of concomitant chemotherapy (p=0.64), and did not correlate with CD20 expression on neoplastic cells (p=0.4).
At univariate analysis the occurrence of ADR correlated with higher hemoglobin levels (p=0.019), higher white blood cell absolute count (p=0.033), lower neutrophils (p=0.01) and monocyte (p=0.026) percentages, higher lymphocytes percentage (p=0.007), splenomegaly (p=0.01), history of allergy (p=0.007), and, in patients with lymphoproliferative disorders, bone marrow infiltration (p=0.005). Interestingly, three groups of patients at different risk for ADR were identified, according to a predictive risk heatmap developed using the combination of four parameters (splenomegaly, history of allergy, hemoglobin levels and gender) selected by multivariate analysis.
In conclusion, ADRs are more commonly observed in patients with lymphoproliferative disorders than in patients with autoimmune diseases and did not correlate with CD20 expression. In the large majority of cases they are easily manageable. A predictive model based on four simple clinico-biological variables may be useful in predicting the development of infusional side-effects, allowing to identify patients at higher risk of ADRs for whom appropriate preventing therapies are needed.

EUROFLOW-BASED IMMUNOPHENOTYPIC CHARACTERIZATION OF CD34+ CELL COMPARTMENT IN JUVENILE MYELOMONOCYTIC LEUKEMIA (JMML): A NEW TOOL FOR DIFFERENTIAL DIAGNOSIS

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Background
Juvenile myelomonocytic leukemia (JMML) is a rare and severe myelodisplastic and myeloproliferative neoplasm of early childhood. Diagnosis is still challenging because clinical manifestations overlap with other diseases. Yet, immunophenotypic characterization is not considered informative and is not included in the diagnostic work-up of JMML. In this report we aimed at the characterization of CD34+ cell compartment using a fully standardized approach developed by Euroflow consortium for immunophenotyping of hematological malignancies to

assess whether this analysis can be of impact in differential diagnosis of JMML.

Methods

We analyzed 19 JMML patients at diagnosis (14 BM, 5 PB and 4 paired samples), 22 control subjects and 5 cases with suspected JMML subsequently not confirmed using the following standardized antibody combinations:
cyCD3/ CD45/ cyMPO/ cyCD79a/ CD34/ CD19 CD7/smCD3
HLADR/CD45/CD16/CD13/CD34/CD117/CD11b/CD10
HLADR/CD45/CD35/CD64/CD34/CD117/CD300e/ CD14
HLADR/CD45/CD36/CD105/CD34/CD117/CD33/CD71

Results

The proportion (mean % ± SD) of granulocytic and monocytic precursors were not significantly different in JMML CD34+ cells as compared to controls being 28.5% ± 15.1% vs 21 % ± 12.8% ($p = 0.13$); 13.4% ± 7.5% vs 12.4% ± 7.1% ($p = 0.7$), whereas erythroid cells were reduced (0.7% ± 1.1% vs 2.8% ± 1.7%, $p<0.001$), respectively. By contrast, B-cell precursors were strongly reduced in JMML as compared to controls (14.5% ± 24.3% vs 51.6% ± 15.7%, $p<0.0001$), while CD7+ lymphoid precursors resulted enhanced (23.1±% ± 17.4% vs 2.5% ± 1.4%, $p<0.0001$).

We consistently found unusual immunophenotypes in the CD34+ subset (CD7+/MPO+ and/or CD79a+/CD7+) in 11/11 analyzed JMML BM cells (mean 7.7% ± 5.9%) while in controls these cells were virtually absent (0.03% ± 0.06%, $p<0.0001$). Five patients that were suspected having JMML showed CD34+ profile not significantly different from that of normal subjects and were finally diagnosed as non-JMML.

BRONCHOALVEOLAR LAVAGE: BETWEEN FLOW CYTOMETRY AND CYTOLOGY

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Bronchoalveolar lavage fluid analysis evaluates the inflammatory and immune state of deep lung. Basically, it's characterized by two steps: the analysis of the immune state by the assess of lymphocytes subsets by flow cytometry (FCM) and the evaluation of the inflammatory state by cytological examination. Increases, sometimes even marked, of the CD4/CD8 ratio may be suggestive of sarcoidosis, or the absent reversal of the ratio can make very unlikely diseases in which this is expected, as in extrinsic allergic alveolitis. Also, FCM is still very useful in lymphoproliferative disorders or in Langerhans cells (CD1+) increase .

Currently there are publications in which additional markers have been used to give also a differential cell count using FCM (usually without mast cells). Actually, obtaining quickly a differential cell count by FCM can't avoid the careful assessment of the specimen by optical microscopy (OM) both from a quantitative point of view (squamous epithelial/ciliated epithelial cells percentage assess the suitability of the sample to represent the cellular pattern of deep lung) than from a qualitative point of view (e.g report erythrophagocytosis, foamy macrophages, acellular globules as in Pulmonary alveolar proteinosis or proliferation of type II pneumocytes wich requires a d.d from adenocarcinoma).

Aim of the work was to quantitatively asses lymphocytes and neutrophils using markers already used for lymphocytes subsets (CD45/CD16+56) by comparing obtained data with those derived from the analisys of the cytospin by OM.

Thirty samples have been analysed and the results were similar with those from other works: overestimation of lymphocytes by FCM compared to OM and a good correlation between the two methods concerning neutrophils.

Conclusions: Although the morphological analysis by OM is still essential for above mentioned reasons, a preliminary quantitative assessment of lymphocites may be useful, usually underestimated by OM probably because of the inequality of cellular distribution in the cytospin (some authors report that lymphocytes are mainly placed on the periphery at the expense of macrophages, in greater or lesser proportion, depending on the speed used). On the contrary the assessment of neutrophils, since there's a good correlations between the two method, may be used as a sort of check of the differential cells count obtained by OM without additional costs.

REGULATION OF HIF-1α IN TP53 DISRUPTED CLL CELLS AND ITS INHIBITION AS A STRATEGY TO OVERCOME FLUDARABINE RESISTANCE

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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 (i.e. TP53 disruption), and to the interactions of CLL cells with stromal cells (SC) of the microenvironment. HIF-1α is a transcription factor implicated in cell adaptation to hypoxia and it is involved in the regulation of genes implicated in tumor progression. In CLL cells HIF-1α is constitutively expressed even in normoxia and modulates the interactions with SC. The aims of this study were to understand the HIF-1α regulatory pathways in TP53 disrupted ($TP53^{dis}$) CLL cells and to evaluate the ability of HIF-1α inhibition to exert a direct cytotoxic effect and to potentiate fludarabine cytotoxicity toward TP53dis CLL cells.

CLL cells were considered $TP53^{dis}$ when TP53 mutation or 17p deletion were present. Otherwise CLL cells were considered wild type ($TP53^{wt}$). CLL cells were cultured

alone or with the M2-10B4 SC under normoxic or hypoxic conditions and exposed to fludarabine and/or the HIF-1α inhibitor BAY 87-2243. We evaluated the activity of ERK1-2, Ras, RhoA, RhoA kinase, Akt by Western Blot or specific immunoassay kit. HIF-1α expression was assessed by RT-PCR and Western Blot. Cell viability was analyzed by AnnexinV/propidium Iodide immunostaining. $TP53^{dis}$ CLL cells showed a significantly higher HIF-1A gene expression compared to $TP53^{wt}$ CLL cells, and also had higher amount of HIF-1α protein. Accordingly, $TP53^{dis}$ CLL cells overexpressed the HIF-1α target genes p21, Bcl-2 and ENO1, and had a more active glycolysis than $TP53^{wt}$ CLL cells. Hypoxia further increased HIF-1α expression in both $TP53^{dis}$ and $TP53^{wt}$ CLL cells. Similarly, the co-culture of CLL cells with SC further upregulated HIF-1α in both subsets via the activation of Akt, Ras- and RhoA-dependent signaling cascades. BAY87-2243 efficiently inhibited HIF-1α and induced a significant reduction in viability of CLL cells in both subsets. *In vitro* fludarabine-resistant CLL cells showed higher levels of HIF-1A gene compared to fludarabine-sensitive, and the inhibition of HIF-1α with BAY87-2243 restored fludarabine-induced cytotoxicity. Lastly, HIF-1α inhibition was also able to reverse the SC-mediated resistance to fludarabine cytotoxicity in both $TP53^{dis}$ and $TP53^{wt}$ CLL cells.
Based on our results, HIF-1α represents an interesting therapeutic target in CLL, in particular for the high-risk disease subset.

BIOLOGICAL AND CLINICAL SIGNIFICANCE OF EXTRACELLULAR VESICLES IN HEMATOLOGICAL MALIGNANCIES

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Extracellular vesicles (EVs) are lipid bilayer particles (30-2000nm in diameter) released by normal and neoplastic cells. According to their size, EVs can be divided in exosomes, microvesicles and apoptotic bodies and are enriched in protein, mRNA, miRNA and DNA. EVs mediate intercellular communication interacting with target cells and controlling fundamental biological functions. In this setting, we first demonstrated that EVs mediate communication between umbilical cord blood CD34+ (UCB-CD34+) and bone marrow mesenchymal stem cells (BM-MSCs). The exposure of UCB-CD34+ to BM-MSC EVs influenced UCB-CD34+ cell fate modifying their gene expression profile and rendering them more viable and less differentiated. Moreover, BM-MSC EVs treatment in in vivo mouse model caused an augmented migration of CD34+ from peripheral blood to BM niche indicating that BM-MSC-EVs could be helpful in BM microenvironment reconstitution in transplant applications. EVs are present in different biological fluids, including serum, and have a potential role as disease biomarkers. We observed that EVs levels are significantly elevated in serum of patients affected by different hematological malignancies (HMs), such as chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML) and Waldenstrom macroglobulinemia, respect to healthy controls. Furthermore, EVs from patients specifically express tumor-related antigens and correlate with different HM clinical parameters. In particular, in CLL we found that absolute number of B-cell derived EVs significantly correlate with high tumor burden. Moreover, absolute MV number cutoff selected by ROC analysis distinguished Rai stage 0 patients with shorter time to treatment (TTT) from those with more stable disease. Likewise, in the entire cohort, 2 groups of patients with different overall survival (OS) and different TTT were identified. At multivariate analysis, serum EVs independently predicted for OS (along with Rai stage) and TTT (along with Rai stage, lymphocytes, CD38). In addition, analyzing their content, we found that the EV miR155 levels are significantly higher in different HM compared to controls. EV miR155 ROC curve analysis reveal significantly different patterns in CLL and AML compared to controls and in AML compared to myelodysplastic syndromes. In conclusion, our data indicate that circulating EVs and EV miR155 could represent new attractive biomarkers in HMs.

CIRCULATING PLATELET AND MEGAKARYOCYTE-DERIVED MICROPARTICLES OF JAK2V617F MUTATED PATIENTS WITH MYELOFIBROSIS ARE DISREGULATED: A NOVEL LIQUID BIOPSY TOOL OF RESPONSE TO RUXOLITINIB?

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Background: Microparticles (MPs) are small vesicles (0.1-1 micron) deriving from plasma membrane budding during homeostasis and cell activation. MPs express antigens and contain constituents from cell of origin and are increased in conditions that are characterized by high cell turnover or death, particularly inflammatory, autoimmune and neoplastic diseases. Myelofibrosis (MF) is a clonal neoplasia of the hematopoietic stem/progenitor cells characterized by distinctive abnormalities in megakaryocyte (MKC) development and platelet (PLT) activation
Aims: This study aims to: 1) enumerate circulating MK and PLT-derived MPs of MF pts; 2) evaluate the effect of RUX on MPs production by PLT and MK; 3) investigate whether circulating MK and PLT- MPs may be a biomarker of response to RUX.
Methods: EDTA-anticoagulated peripheral blood from healthy donors (HD, n=10) and JAK2V617F positive MF pts (n=12) at intermediate-2/high IPSS risk was collected at baseline and 3 and 6 months after RUX therapy and immediately centrifuged. PLT (CD61+CD62P+) and MK (CD61+CD62P-)-derived MPs were analysed in PLT poor plasma samples by flow cytometry (CytoFLEX, Flow Cytometer-Beckman Coulter). The instrument was calibrated with MEGAMIX Beads (Beckman Coulter) with various diameters to cover the MPs (0.5-0.9 μm).
Results: At 3 and 6 months, 5 out of 12 pts achieved a spleen response (R) according to 2013-IWG-MRT criteria. At baseline, the mean percentage of MK-derived MPs was significantly decreased (29±6 vs 72±5; $p<0.0001$) while that of PLT-derived MPs significantly increased (49±7 vs 11±1; $p<0.0001$) in MF pts compared to HD.

However, the mean percentage of MK-derived MPs from pts not achieving a spleen response (NR) was significantly decreased compared to R (17±6 vs 45±5; p<0.005) and HD (17±6 vs 72±5; p<0.0001). By contrast, the mean percentage of PLT-derived MPs was significantly increased in NR compared to R (64±7 vs 37±9; p<0.05) and HD (64±7 vs 11±1; p<0.001). Of note, NR pts had significantly lower PLT number as compared with R pts (220±29 vs 422±98 p<0.05).

Summary/Conclusion: At variance with HD, the majority of circulating MPs in JAK2V627F mutated MF pts at intermediate-2/high IPSS risk derived from PLTs. RUX therapy did not modify the MK/PLT-derived MPs pattern, suggesting that JAK1/2 inhibition does not seem to affect the pathways of MK/PLT MPs production or clearance. Most importantly, MPs evaluation at baseline is significantly associated with subsequent spleen response.

CD81 NEGATIVE EXPRESSION PREDICTS A POOR CYTOGENETIC AND PROGNOSTIC RISK GROUP IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Several factors can predict the outcome in chronic lymphocytic leukemia (CLL), including immunophenotipic features. The expression of CD49d and CD38 represented the most important prognostic markers in CLL but no further markers have been investigated between those newly used. On the other hand, no markers seem to predict cytogenetic aberrations. CD81 is a tetraspasmin widely espressed on B cells and weakly expressed on CLL cells. Immunophenotipic studies of CD81 expression in patients with CLL are scanty and its value in predicting cytogenetics lesions as well as the outcome remain unknown. Thus, the aim of this study was to evaluate the potential role of CD81 in indentifying different cytogenetic and prognostic risk groups in CLL. Samples of bone marrow from 71 patients with CLL were investigated for the surface expression of CD81 reported as percentage of CLL cells expressing the antigen and MFI. Thirty-one patients were treated and studied for minimal residual disease (MRD). The best cut-off points for CD81 was sought by constructing ROC curves, so that values greater than 20% of CLL cells and 530 by MFI were considered as positive samples (CD81 pos) while the expression below these levels defined negative samples for this marker (CD81 neg). CD81 neg samples were significantly associated with unfavorable cytogenetic aberrations (67% vs 33%) such as 17p and 11q deletions while CD81 pos samples were associated with favorable cytogenetic aberrations (96% vs 4%) such as 13q deletion and +12 trisomy (p=0.000). Majority of patients showing a negative cytogenetic profile with respect to previous aberrations had CD81 pos CLL cells (72% vs 28%)(p=0.000). There was an agreement between CD81 neg and CD38 positive expression(p=0.002). No significant correlations were found between CD81 expression and clinical features of CLL. When the only patients who underwent chemotherapy were considered, a significant association was showed between CD81 neg patients and a positive MRD (p=0.015). Interestingly, CD81 neg patients showed a significantly lower disease free survival (DFS) than those CD81 pos (p= 0.038).No significant associations were found between CD81 expression and the overall survival. In conclusion, the absence of CD81 expression on CLL cells identifies patients who had unfavorable cytogenetic aberrations and a lower DFS compared to patients with positive CD81 expression, thus confirming the predictive role of CD81 in CLL.

IMMUNE RECONSTITUTION AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA: INFLUENCING FACTORS AND PREDICTIVE VALUE

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Post tranplant dysregulation of the immune system limits the success of hematopoietic stem cell transplantation (HSCT). 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. Hence, we prospectively investigated post transplant changes in lymphocytes subsets, hematogones (HGs) and dendritic cells (dendritic plasmocytoid cells (DPCs) and dendritic myeloid cells (DMCs)) in bone marrow (BM) samples of AML patients and their associations with minimal residual disease (MRD), GvHD, infections and HSCT type. Then we evaluated which of the immune cell subset may influence the survival. 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. Immune subsets as well as MRD were investigated using 6-color flow-cytometry. Thresholds for each immune cell subset were obtained from a comparison with healthy control BM (n=15). NKs displayed values higher than 2.21%, 2.56% and 2.45% at +1, +3 and +6 mths from transplant, respectively. T-CD4+ were less than 4.6% at each time point while T-CD8+ 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. Only DPC showed counts lower than 0.20% and 0.22% at +3 and +6 mths. A significant association was found between patients experiencing aGvHD and NK cells over 2.21% at +1 month (p=0.05). Similarly, patients undergoing sibling-HSCT and not experiencing cGVHD showed HGs greater than 1.55% (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). Our results suggest a role of NKs in the pathogenesis of aGvHD. Transplant donor 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.

CHARACTERIZATION OF THE ADENOSINERGIC AXIS IN THE EM-TCL1 CHRONIC LYMPHOCYTIC LEUKEMIA MOUSE MODEL

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Background: the neoplastic niche is a closed environment where tumor cells interact with surrounding non-neoplastic elements. Convincing data from independent groups have shown that this environment is characterized by low oxygen tension, overexpression of ATP-metabolizing enzymes and increased extracellular ATP and adenosine levels. Extracellular adenosine can be generated from ATP and/or ADP through the sequential action of the ectoenzymes CD39 and CD73. Adenosine elicits its physiological responses by binding to and activating one or more of the four transmembrane type 1 purinergic receptors. Specifically, in the tumor context, binding of adenosine to the A2A receptor results in potent cytoprotective and immunosuppressive effects. We previously focused on chronic lymphocytic leukemia (CLL), a disease characterized by the progressive expansion of a mature population of B lymphocytes, showing that a subset of patients with a $CD73^+$ clone has a poor prognosis. This patient subset can actively generate adenosine, which favors leukemic cell survival, synthesis of immunosuppressive cytokines, while inhibiting T cell proliferation.
Aim of the work: the main aim of this work is to characterize the adenosinergic axis *in vivo*, determining the impact of this enzymatic machinery both in CLL progression and modulation of immune responses. To this purpose, a mouse model based on the adoptive transfer of spleen-purified leukemic cells, derived from TCL1 mice, in naïve C57BL/6 immunocompetent recipients was adopted. Leukemic cells rapidly engraft into the spleen, the bone marrow, the peritoneal cavity and the peripheral blood. Disease progression was monitored by cytofluorimetric analysis for the presence of a $B220^+/CD5^+$ leukemic population in the blood.
Results: the expression of the adenosinergic axis, both on the leukemic side as well as on T lymphocytes, was firstly assessed, by comparing cells obtained from the spleen of TCL1 and $C57BL/6^{WT}$ mice. Flow cytometry analysis of $B220^+/CD5^+$ leukemic cells indicated that CD39 is widely present at high levels, while CD73 is heterogeneously expressed. In contrast, the CD26 adenosine deaminase-anchoring protein is poorly detected. These data recapitulate the phenotype observed in human CLL patients and sustain the hypothesis of variable levels of adenosine in tissues colonized by leukemic cells. Surface expression of CD39, CD73 and CD26 was also evaluated on T cells. Results indicated that both $CD4^+$ and $CD8^+$ lymphocyte subsets in TCL1 mice are highly $CD39^+$ compared to the WT counterpart, as opposed to CD73 that is expressed at low levels. No significant differences where highlighted for CD26. These results were confirmed by quantitative RT-PCR analysis on sorted B, $CD4^+$ and $CD8^+$ T lymphocytes.
The development of CLL disease is usually associated with specific T cell and myeloid subsets changes. To address this point, attention was focused on the characterization of these cell populations and the effects on their polarization due to the presence of leukemic cells. Multiparametric flow cytometry analyses were adopted to identify naïve ($CD44^-/CD62L^+$), memory ($CD44^+/CD62L^+$), central memory ($CD62L^+/CCR7^+$), effector memory ($CD62L^-/CCR7^-$) and effector ($CD44^+/CD62L^-$) as well as inflammatory ($Ly6C^{high}/CD43^{low}$), intermediate ($Ly6C^{high}/CD43^{high}$) or patrolling ($Ly6C^{low}/CD43^{high}$) monocytes. Data obtained confirmed that in TCL1 mice the naïve T cell subset is dramatically reduced compared to WT mice, with the simultaneous increase in memory as well as exhausted sub-populations (e.g., effector and effector memory). Also the monocyte compartment was affected by the presence of leukemic cells as inferred by a reduction of the inflammatory subset, which usually migrate to inflammatory sites, with a parallel increase of patrolling population, generally involved in tissue regeneration after inflammation.
Conclusions: taken together, these preliminary results showed that the adenosinergic axis is over-expressed in the TCL1 mouse model and confirmed that the presence of leukemic cells polarize the host immune cells, favoring the establishment of a tumor supportive environment. They also argue in favor of further studies using specific inhibitors of this signaling pathway.

EXTENSIVE FLOW CYTOMETRY ANALYSIS TO EVALUATEIMMUNOLOGICAL RECONSTITUTION-FOLLOWING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background: Allogeneic hematopoietic stem cell transplantation (ASCT) representa standard treatment for differenthematologic malignancies. Following transplantation, regain of function of the donor-derived immune system is crucialfor allclinical outcomes; however, complete immunological reconstitutionmay requiremonths to years, both being affected and leadingto potentially severe complications, including graft versus host disease (GvHD) and viral infections/reactivation.
Methods: Aiming to prospectively evaluate immunological reconstitution and possible association with clinical outcomes, blood samples from all patients undergoingSCT in our Centersince December 2014 wereanalyzed at baseline (before ASCT)and at different time points (+1, +2, +3, +6, +9,+12, +24 months)after allo-SCTby extensive flow cytometryassays including: *double positive* ($CD4^+CD8^+$) *thymocytes*; *naïve*($CD45RA^+CCR7^+$), *effector* ($CD45RA^+CCR7^-$), *central memory*-T_{CM} ($CD45RA^-CCR7^+$), *effector memory* -T_{EM} ($CD45RA^-CCR7^-$) $CD4^+$and $CD8^+$ T cells; regulatory($CD25^{bright}CD127^{low}$) $CD4^+$ T cells; *naïve* ($CD27^+IgD^+$), *IgM-memory* ($CD27^+IgD^+$) and *switched-memory* -SM($CD27^+IgD^-$) $CD19^+$B cells; *regulatory* ($CD3^-CD56^{bright}CD16^-$) and *cytotoxic*($CD3^-CD56^{dim}CD16^+$) Natural Killer (NK) cells.
Results: 27 patients(median age 53 yrs,range 19-66 yrs)were included in the study. Indication for ASCT was AML in 14, ALL in 6, MDS in 3 and NHL in 4. NK were the first cell subset to return to a normal level after transplantation, with an early expansion of regulatory cells during the 1^{st} month and a subsequent expansion of cytotoxic

cells from the 2nd monthon. With regard to T cells, we observeda progressive peripheral expansion of the mature subsets (especially T_{EM}) from the 2nd month on, probably originating from donor mature T cells, with a subsequent maintained prevalence of the $CD8^+$ over the $CD4^+$ cells.The reconstitution of $CD4^+$ and $CD8^+$ *naïve* T cells from donor hematopoietic cells through the thimic pathway was delayed, startingto increase only from the 9th monthandleading to a long-lastinglimited T-cell repertoire. B cell tended to return tonormal counts within 9 months, mainlythanks to naïve cells, while memory and SM cells remained low up to 2 years after ASCT.
Conclusion: Compared to standard analysis, extensive-flow cytometric analysis allows to study specific immune reconstitution after SCT, confirmingthat the achievement of a full immunocompetent state requires a long period of time.

A RETROSPECTIVE MONOCENTRIC STUDY ON BREAST IMPLANT-ASSOCIATED ANAPLASTIC LARGE CELL LYMPHOMA (BI-ALCL)

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BI-ALCL is a rare anaplastic ALK-negative T-cell lymphoma recently recognised by the WHO classification of lymphoid neoplasms. Although rare (0.04-0.5% of all breast malignancies), the estimated risk of developing BI-ALCL is 18-67 times higher in women with breast implant than without implant. The disease usually manifests as late periprosthetic effusion (seroma) where CD30+ large pleomorphic cells with incomplete cytotoxic T-cell phenotype can be detected by cytology and immunohistochemistry (IHC). In situ cases have excellent prognosis and can be treated by surgical removal of the prosthesis and the surrounding capsule, whilst chemotherapy (CT) is recommended for occasional infiltrative disease. Until February 2017, 359 BI-ALCL cases have been recorded by FDA, 25 of which in Italy, but the tumour may be currently underdiagnosed. Due to the limited number of cases so far reported, a uniform and standardised clinical-diagnostic approach to the disease is still lacking.
We performed a retrospective study on 25 suspected BI-ALCL patients referred to our hospital between July 2011 and July 2017, with the aim to apply appropriate diagnostic tools for a correct classification of this new neoplastic entity and contribute to standardise the diagnostic process.
Eighty-one samples including seroma, periprosthetic capsule, bone marrow, skin, breast nodule and lymph node were evaluated by microbiology, cytology, IHC and, in several cases, flow cytometry (FC) and molecular biology techniques.
Two of 25 patients (accounting for 8% of the national and 0.6% of the global series), who presented with late seroma after surgical reconstruction, were scored positive for BI-ALCL, with medium to large CD30+ ALK- atypical cells depicted in the periprosthetic fluid. In both cases, FC identified a cluster of abnormal lymphocytes with CD45+ CD30+ HLADR+ CD25+ CD4+ CD3- immunophenotype. TCR gene rearrangement was investigated in one case, turning out monoclonal. Both patients were treated with surgery. One of them showed capsule infiltration and received CHOEP CT, achieving complete remission. Only 1 of these 2 cases was recorded in the Italian database.
Our study highlights the importance of applying all available laboratory tools to correctly diagnose this rare tumour, but also gives evidence of heterogeneity in the clinical-diagnostic approach, even within a single centre. An algorithm is therefore proposed to optimise the clinical management of BI-ALCL samples and patients.

COMPARATIVE ANALYSIS OF IMMUNE RECONSTITUTION SHOWS BETTER RECOVERY OF B, NK AND PLASMACYTOID DENDRITIC CELLS AFTER ALLOGENEIC CORD BLOOD AS COMPARED TO PHERIPHERAL BLOOD STEM CELL AND BONE MARROW TRANSPLANTATION

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Introduction: The reconstitution of different immune cell subsets after hematopoietic stem cell
transplantation (HSCT) occurs at different time points and different factors can contribute to delay
this reconstitution. We have studied the impact of several factors on immune reconstitution (type of
disease, conditioning regiment therapy, type of stem cell source, Graft Versus Host Disease (GVHD) and drug therapy used).
Patients and methods: We have studied 503 adult patients receiving pheripheral blood stem cell
(PBSC)(n=262), bone marrow (BM)(n=191) or cord blood (CB) (n=48) transplant at the S. Orsola
Hospital. Data were collected between 2000 and 2014. We have analysed the immune cell subsets in the peripheral blood at specific time points from 1 month up to 1 year after transplant. A total of 1368 samples were processed. Multicolor flow cytometry was employed to measure the numbers of circulating T and B lymphocytes, NK cells and APC subtypes (monocytes, plasmacytoid (pDC) and mieloid (mDC) dendritic cells).
Results: The recovery of pDC at 3 through 12 months after transplant was significantly increased in
patients receiving CB as compared to both PBSC and BM transplants [7 x10e6/l (2.4-11.7) at 3 months after transplant in CB recipients vs 3x10e6/l (1.3-5.7) and 2.9x10e6/l (1.3-5.7) respectively in PBSC and BM recipients] (p 0,0001). Moreover we have confirmed the increase of B and NK cell recovery and the delay in T cell recovery in CB transplants. The only other factor affecting immune recovery was the chronic GVHD. The number of pDC was reduced in patients with extensive cGVHD compared to patients with limited GVHD or without cGVHD at 3 months [1.9 x10e6/l (0.6-4.1) vs 4.1 x10e6/l (2-7.1) and 2.5 (1.1- 5) respectively](p<0,001) up to 1 year. The recovery of T cells and NK cells was unaffected by cGVHD, whereas the B cell recovery was also reduced by cGVHD (significative value only at 9 months).
Conclusion: Our study shows that patients undergoing CB transplantation have better reconstitution of plasmacytoid DC, as well as NK cells and B cells, as compared to patients receiving PBSC and BM grafts. Furthermore, pDC recovery was significantly delayed in patients with cGVHD

CRYOPRESERVATION DOES NOT REDUCE THE IN VITRO INHIBITORY FUNCTION OF GMP- ISOLATED HEALTHY REGULATORY T CELLS

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Using TREGs in immunotherapy may be beneficial in several immune mediated diseases, as Graft Versus Host Disease (GVHD), through induction of immunologic tolerance. The possibility of cryopreserving TREGs might lead to the administration of multiple doses at different time point, thus potentially increasing their efficacy in chronic diseases. However, there are few and controversial data on the functionality of TREGs after cryopreservation. Here, we evaluated the phenotype and the inhibitory capacity of thawed TREGs. TREGs were purified from leukapheresis of normal donors (N=3) by double immunomagnetic depletion (CD8 and CD19) followed by CD25 enrichment using the CliniMACS system (Miltenyi Biotec) under GMP condition. The cells were cryopreserved in saline solution containing 10% Human Serum Albumin (HSA) and 10% DMSO with a controlled-rate freezing. Cell viability was assessed by 7-AAD staining. Number/phenotype and function were evaluated on fresh and thawed TREGs. Cryopreserved autologous T effector (Teff) cells were used in MLR assays.
Before cryopreservation the TREGs enriched product mean viability was 95±4% and the mean percentage of CD45+CD4+CD25+CD127low and CD45+CD4+CD25+CD127lowFoxP3+ cells was 74±13% and 66±10%, respectively.
We then analysed the TREGs enriched product after thawing. Mean viability of thawed TREGs, by 7-AAD staining, was 85±7%. The viable TREGs were almost totally CD4+CD25+ (97±2%). The mean percentage of CD4+CD25+CD127low and CD4+CD25+CD127lowFoxP3+ thawed cells was 73±14% and 71±20% respectively. The contaminant cells present in the TREG enriched product were mostly CD4+CD25+CD127+ (around 18%). We further characterized the phenotype of the CD4+CD25+CD127low population. This population was almost totally Foxp3+ (93±6%) and expressed selected markers at various degree (CD62L (50±2%), CD15s (6±2%), CD45RA+ (19±3%), HLA-DR+ (15±10%), CCR7+ (74±5%), CD49d (52±14%), CD26+ (1±0.4%), CD196+CD161+ (4±1%).
Notably, viable thawed TREGs were able to induce inhibition of autologous Teff cells in a 1:2 Tregs:Teff ratio as freshly isolated TREGs: 44±16% (thawed) vs 55±24% (fresh) of inhibiton ($p > 0.1$).
In conclusion, here we demonstrated that thawed TREGs from healthy donors mantain a stable phenotype. In addition, in our hands TREGs show good suppressive ability after thawing despite lower expression of CD62L and CD15s (markers of most suppressive TREGs) as compared with the available published data (Florek et al 2015; Miyara et al 2015).

A NOVEL NF-KB INHIBITOR SHOWS ACTIVITY IN CHRONIC LYMPHOCYTIC LEUKEMIA AND RICHTER SYNDROME

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Background: Chronic lymphocytic leukemia (CLL) cells survival is critically dependent on heterotypic interactions with non-malignant cells in the microenvironment. Most of these signals induce the activation of the transcription factor NF-κB, that in turn controls complex cellular functions, including apoptosis, cell survival and proliferation. Given its central role in tumorigenesis, NF-kB represents an attractive target, considering also the current absence of approved selective drugs.
Aim of the work: The aim of this work is to test the efficacy of IT901, a recently reported novel NF-kB inhibitor, in CLL and in its more aggressive transformation, Richter syndrome (RS), a still clinical demanding disease. The molecular mechanisms of action of the drug in leukemic cells are studied, both in vitro and in vivo, exploiting primary cells and xenograft models, alongside its effects on cells belonging to CLL microenvironment.
Results: By flow cytometry analysis, we showed that IT901 induces apoptosis in primary leukemic cells in a dose- and time-dependent manner, showing significant efficacy starting at 24h of treatment. The apoptotic response is independent of the clinical prognostic subgroup. Conversely, IT901 has minimal impact upon normal B and T lymphocytes. Moreover, treatment of CLL cells with IT901 significantly interferes with NF-kB transcriptional activity, with a diminished binding of both p50 and p65 subunits to DNA, as inferred by ELISA assays. These results were confirmed at the biochemical level, showing a diminished expression of these subunits in the nucleus, as well as of the whole NF-kB complex in the cytoplasm. At the molecular level, compromised expression of NF-kB triggers activation of the Caspase-3 apoptotic pathway, with increased expression of pro-apoptotic proteins (e.g., Bim), paralleled by a diminished expression of the anti-apoptotic ones (e.g., XIAP). Concomitantly, treatment of primary CLL cells or CLL-like cell-lines, resulted in a prominent increase in mitochondrial reactive oxygen species (mROS), as measured by using a specific MitoSOX Red indicator for live-cell imaging.
A critical role for disease progression and for providing protection from drug-induced apoptosis is played by the CLL microenvironment. Thus, it is important to consider the effects of novel drugs also on non-neoplastic bystander elements. Nurse-like cells (NLCs) are a population of monocyte-derived activated macrophages that sustain CLL cells through soluble factors and direct cell-contact mechanisms. This interplay is able to induce activation of the NF-kB signaling in both cellular compartments. Consistently, IT901 was able to inhibit nuclear localization of the p65 subunit in NLCs and impaired NF-kB-driven expression of molecules involved in the cross-talk with CLL cells and in immune responses (e.g., CD49d, ICAM-1, CD274), essential elements in creating a pro-leukemic niche. The consequence is that accesso-

ry cells do not protect leukemic cells from IT901-induced apoptosis.
IT901 was also effective in primary cells from patients with Richter syndrome. Its anti-tumor properties were then confirmed in xenograft models of CLL and in RS patient-derived xenografts (PDX), with documented NF-kB inhibition and significant reduction of tumor burden.
Conclusions: Altogether, these results indicate that IT901 is able to interfere with the NF-kB activity, both in leukemic cells inducing apoptosis, and in bystander cells, interrupting the support that CLL obtains from the microenvironment. In conclusion, they provide a preclinical proof of principle for IT901 as a potential new drug in CLL and RS.

TARGETING HIF-1A AND ITS REGULATORY PATHWAYS AS A STRATEGY TO HAMPER TUMOR-MICROENVIRONMENT INTERACTIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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The CXCL12/CXCR4 axis has a fundamental role in the microenvironment-mediated protection of chronic lymphocytic leukemia (CLL) cells from spontaneous and drug-induced apoptosis. The binding of CXCL12 to CXCR4 activates RhoA- and Ras-dependent signalling. We have previously shown that co-culture with stromal cells (SC) induces in CLL cells the activation of RhoA/RhoA kinase and Ras/ERK1-2 signalling, the upregulation of Akt, and an increased activity of the transcription factor HIF-1α. The purpose of this study was to identify new possible pharmacological targets involved in the CXCL12/CXCR4 axis in order to impair the protection exerted by SC towards spontaneous and fludarabine-induced apoptosis in CLL cells.
Patient-derived CLL cells were cultured alone or with murine M2-10B4 SC. Patient-derived bone marrow SC were also generated. In selected experiments, recombinant CXCL12, CXCR4 inhibitor AMD3100, fludarabine, simvastatin, ERK1-2 kinase inhibitor PD98059, HIF-1α inhibitor BAY87-2243, or PI3K-delta inhibitor idelalisib were added. We analysed the activity of Ras, RhoA, RhoA kinase, Akt and HIF-1α, the expression of ERK1-2, the expression and phosphorylation of HIF-1α, the CXCL12 production, and CLL cell viability.
The exposure of CLL cells to recombinant CXCL12 led to the activation of RhoA- and Ras-dependent signalling, and to the downstream upregulation of HIF-1α. The CXCR4 antagonist AMD3100 completely abrogated the positive regulation exerted by both CXCL12 and SC, thus unveiling the key role of the CXCL12/CXCR4 axis in the SC-induced modulation of these signalling pathways. The inhibition of Ras and RhoA activity by simvastatin, the inhibition of ERK1-2 and HIF-1α by PD98059 and BAY87-2243, and the targeting of the PI3K/Akt pathway with idelalisib effectively blocked the SC-induced expression and activity of HIF-1α, significantly impairing the SC-mediated protection of CLL cells, both in absence or presence of fludarabine.
At the SC level, simvastatin and BAY87-2243 effectively inhibited HIF-1α expression both in M2-10B4 and in patient-derived SC. Moreover, simvastatin significantly reduced the secretion of CXCL12, which is a known transcriptional target of HIF-1α.
Our data demonstrate that the targeting of HIF-1α and its regulatory pathways - both at the tumor cell and at the SC level - is an appealing strategy to overcome the microenvironment-mediated protection toward spontaneous and fludarabine-induced apoptosis in CLL cells.

A CASE OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) WITH ATYPICAL TRISOMY 12: A CYTOGENOMIC STUDY

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We report on a 61-year-old man who was affected by a refractory Chronic Lymphocytic Leukemia (CLL). On clinical diagnosis, FISH (Fluorescence In Situ Hybridization) panel, perfomed at an other center, revealed trisomy 12 (+12) and p53 gene deletion in 60% of the nuclei, while ATM gene and chromosome (chr) 13 were normal. Mutational studies for IgVH and TP53 showed wild type conformation. From 2007 to June 2012 he was treated with different courses of chemotherapies (fludarabine+ciclofosfamide+rituximab, endoxan, leukeran, R-bendamustine) without response. On October 2012, at the time of our first visit, FISH and cytogenetic analysis were carried out on peripheral blood. Interphase FISH analysis revealed +12 in 53% of the nuclei, chr 13 deletion in 68% of the nuclei, "normal" ATM, and p53 gene deletion in 72% of the nuclei. Cytogenetic analysis, performed on B-lymphocytes after ODN DSP30+Interleukine 2 stimulation showed different clones with numerical and structural alterations, involving chrs 3, 12, 13, 17, Y, and different marker chrs. There was no evidence of trisomy 12. To investigate the discrepancy on +12 between FISH and stimulated cytogenetic we performed an a-CGH study, using SureScan Microarray Scanner G2600D platform (Agilent Technologies), with slides SurePrint G3 ISCA CGH-SNP Bundle, 4x180K. Data analysis was performed by CytoGenomics 3.0.6 software. aCGH revealed a 8 Mb deletion on short arm of chr 3, a 5 Mb deletion on long arm of chr 13, deletion of the short arm of chr 17, –Y, and on chr 12 the amplification of the short arm, centromeric region and some regions of the long arm. This was the reason why trisomy 12 was criptic to cytogenetic analysis. To establish candidate genes involved in the CLL pathogenesis and refractoriness to treatments the Genome Browser UCSC was used. The region 12q15 carries an important gene, MDM2 (Murine Double Minute 2), which amplification is known to be a potent inhibitor of protein p53 oncosuppressor activity. Unfortunately, the patient died in 2014 for infective complications. Recently, in 2016, MDM2-antagonist molecule, RG7112, has been proposed in phase I studies on cohorts of patients with chronic and acute leukemias, with good results as it restores p53 oncosuppressor function.

Immunology

NEUTROPHIL ANTIBODY DETECTION IN SERA OF NEUTROPENIC PEDIATRIC PATIENTS BY MICROBEADS COATED WITH HUMAN NEUTROPHIL ANTIGENS

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Introduction Neutrophil antibodies (abs), with prevalent specificity against Human Neutrophil Antigens (HNA) are implicated in Autoimmune Neutropenia (AIN) and in Neonatal Alloimmune Neutropenia (NAIN). The Flow Cytometry-Granulocyte Immunofluorescence Test (flow-GIFT) and the Granulocyte Agglutination Test (GAT) are the current gold standard methods for the detection of HNA abs. However, flow-GIFT shows a long execution time and limited sensitivity, particularly if not performed on a panel of HNA typed neutrophils including homozygous HNA-1a (the most common specificity involved in primary AIN) and -1b typed donors bearing a high antigen density. Recently, a method based on microbeads coated with purified HNA antigens (LabScreen Multi, LSM, One Lambda) has been proposed for the detection of circulating HNA abs. In this study we compared the results of LMS with flow-GIFT in sera of suspected AIN (n=88), NAIN (n=8) and in 11 positive control (PC) sera (CQ INSTAND). **Methods** Flow-GIFT was performed against a 4-cell panel including one homozygous HNA-1a and -1b healthy donors. FACSCantoII and FACSDiva software (BD Biosciences) were used for sample acquisition and analysis. LSM was performed according to the manufacturer's instructions using in-house cut-off values. **Results** Flow-GIFT was positive in 42 sera from suspected AIN (47,7%); LSM was positive in 19 of the latter (45,2%). Nine (19,5%) of 46 sera negative by flow-GIFT resulted positive by LSM, with HNA-1a or HNA-1b specificity. Six out of 8 mother' sera from suspected NAIN cases showed concordant results by the two methods. In particular, 3 sera resulted positive for HNA-1a or HNA-1b abs and 3 sera were negative with both methods. In the remaining 2 cases only flow-GIFT identified mother's abs not specific for newborns' antigens. Flow-GIFT identified all abs specificities in 11 PC sera tested. LSM correctly identified 8 PC sera; for the remaining 3 sera, in 1 missed HNA-3a abs, whereas in 2 detected both HNA-3a and -3b abs. **Conclusions** LSM correlated well with flow-GIFT for allo-abs detection, whereas for auto-abs seems to detect well only specific HNA abs, but not pan-reactive or cross-reactive neutrophil abs. Detection of HNA-3a abs has to be improved. The use of beads coated with well defined antigen densities could overcome problems due to limited availability of cell panels from typed homozygous donors. Moreover, it may reduce the time of screening execution and could improve test standardization.

CLINICAL RESPONSE TO USTEKINUMAB IN SEVERE ERYTRODERMIC PSORIASIS CAUSED BY CARD-14 MUTATION

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Autosomal dominant gain of function mutations in caspase recruitment domain family member 14 (CARD14) were found to cause plaque psoriasis in two families (30% also developed psoriatic arthritis) and severe generalized pustular psoriasis as a monogenic form of childhood (CARD14-mediated psoriasis, CAMPS). CARD14 mutations lead to enhanced NFkB signaling, inducing abundant cytokine production with consequent recruitment of inflammatory cells. Ustekinumab is a human monoclonal antibody directed against the shared p40 subunit of both IL-12 and IL-23, key mediators of psoriasis that are able to stimulate two emerging Th-cell subsets of CD4 + T cells (Th17 and Th22).
Patients: the probands are two 7 year-old twins suffering from early-onset erytrodermic psoriasis first localized and then diffuse over all the skin surface. These cutaneous lesions are severely itchy and associated with onychodystrophies and fissures in both children, and ectropion in the twin sister. There are 3 pairs of twins in the family, 5 of them presenting psoriasis and 2 of them presenting psoriatic arthritis. The children presented poor clinical response to topic and systemic therapy with antihistamine, steroid, retinoid, cyclosporine and etanercept.
Objectives: i) to describe the case of a family presenting an unusual form of severe erytrodermic psoriasis without pustulosis in which whole exome sequencing (WES) analysis revealed a novel CARD-14 mutation; ii) to report the clinical and immunological response to ustekinumab.
Results: WES analysis revealed in the affected members a novel missense mutation of CARD-14 gene (c.446T>G, leading to the missense aminoacid substitution p.L149R). We have treated the children with ustekinumab at dosage of 0.75 mg/kg at week 0 and 4 and every 12 weeks. At week 4 and and 16 both twins showed a dramatic improvement of their clinical conditions, with no significant collateral effect. Then, we have evaluated IFNγ, IL-17A and IL-22 producing CD4+ T cells following peripheral blood mononuclear cells (PBMCs) stimulation with PMA/ionomycin by intracellular cytokine assay. IL-17A and IL-22 levels of stimulated PBMCs at week 4 and 23 were decreased after ustekinumab treatment, consistent with the clinical findings.
Conclusion: gain of function CARD14 mutations can give rise to unusual clinical phenotype like diffuse erytrodermic psoriasis and can be associated to arthritis. Ustekinumab could be a powerful therapeutic option for this singular form of disease.

PHENOTYPIC, MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF THE HUMAN MICROGLIAL CELL LINE, HMC-3

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Microglia, the brain resident macrophages, represent the first line of defense of the central nervous system. By constantly monitoring the extracellular environment, these cells are able to rapidly detect changes and to become activated accordingly. This activity is crucial for the brain homeostasis during development and in the adult brain. However, chronic microglial activation is a common pathogenic mechanism in several neurodegenerative diseases. Most of the evidence on microglial biology rely on rodent experimental models, thus are limited by the significant differences in the molecular evolution of the immune response. Recently, the American Type Culture Collection (ATCC) made available the human microglial cell line, HMC-3 (ATCC CRL-3304). The cell line was established in 1995, through SV40-dependent immortalization of a human embryonic brain-derived primary microglia culture. Cells were characterized as positive for the microglia/macrophage marker Iba-1, whereas other lineage markers (*i.e.* CD14 and CD68) showed differential expression depending on the technique used. In addition, they spontaneously release sizable amount of free oxygen radicals (ROS), which is increased by the HIV-1 Tat C protein. More recently, data obtained with the HMC-3 cell line were confirmed in primary cultures of human microglial cells, thus suggesting that this is a valid model to study human microglia properties. Consistently with the literature and the ATCC data sheet, we have found the HMC-3 cells being Iba-1 positive and GFAP negative under basal conditions, whereas the activation marker MHC-II was only slightly induced by IFNγ (36 h, 10 ng/ml). However, the expression of Iba-1 was not homogenous within the culture, and two populations of cells were identified (49.8 % Iba-1 high and 42.8 % Iba-1 low). Exposure of HMC-3 cells to a pro-inflammatory cytokine mixture consisting of 10 ng/ml IL-1β and 10 ng/ml IFNγ significantly increased the expression of Iba-1 (72.1 % Iba-1 high). The cytokines increased ROS production by 50% and the rate of microglial proliferation. Interestingly, the anti-inflammatory cytokine IL-4, which induces an alternative (M2) pattern of macrophage/microglial activation, differentially modulated Iba-1 expression. Taken together, these data suggest that the HMC-3 cells are viable and can possibly be polarized towards different phenotypes of activation.

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CIRCULATING T REGULATORY CELLS, T FOLLICULAR REGULATORY CELLS AND T FOLLICULAR HELPER CELLS ARE ALTERED IN MYASTHENIA GRAVIS PATIENTS AND MODULATED BY CORTICOSTEROID THERAPY

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Introduction. Myasthenia gravis (MG) is an autoimmune disease caused by the presence of autoantibodies directed against the muscle membrane at the neuromuscular junction. MG is often associated with thymic follicular hyperplasia containing numerous germinal centers mostly composed of activated B cells. At least three T cell populations are likely contributing to B cell activity in MG: T regulatory cells (Treg) and T follicular regulatory (TFR) cells (both populations acting as B cell suppressors) and T follicular helper (TFH) cells that help B cell development. Decreased circulating Treg and TFR cell frequency, and increased circulating TFH cell frequency have been described in MG patients.
Objective. To compare Treg, TFR cell and TFH cell frequency (whole population and subsets) in MG patients (n=9, all with early onset MG, EOMG, untreated) and sex- and age-matched healthy subjects (HS), and to assess the modulatory activity of corticosteroid therapy (CT) routinely administered to attenuate disease activity, on these T cell populations.
Methods. Peripheral blood mononuclear cells (PBMC) were obtained from each patient at diagnosis and upon CT and maintained cryopreserved. PBMC from the two time points and from sex- and age-matched HS were analyzed in the same experimental session to reduce variability. A 11-color flow cytometer was used to determine the frequency of Treg and major Treg subsets (defined by the coordinate expression of $CD3^+CD4^+CD25^+CD127^{low}$ and CD45RA as memory, naïve and activated cells), and of TFH cells and various TFH and TFR cell subsets (defined by the coordinate expression of $CD3^+CD4^+CXCR5^+CD45RA^{dim}$, PD1, CCR6, CCR7, CXCR3, CD127 and CD25).
Results. Compared to HS, untreated EOMG patients showed a significantly lower naïve Treg frequency and a trend toward higher resting TFH cell frequency, mostly reflecting TFH type 2 increase. TFR cell frequency was similar in EOMG patients and HS. CT did not affect TFR and total Treg frequency, although it did diminish memory and activated Treg frequency. TFH cells were diminished by CT, the effect being particularly evident on the effector-like TFH cell subsets.
Conclusion. Our results point to alterations in the effector and regulatory arms of the immune system in EOMG patients, both likely contributing to MG pathogenesis. Changes in TFH cells after CT conceivably contribute to clinical remission. The impact of changes in Treg after CT remains to be clarified.

TOPIC: Neuroimmunology

MICROGLIA ALTERATIONS AND BRAIN DEVELOPMENT DEFECTS IN TREM2-DEFICIENT MICE

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Triggering receptor expressed on myeloid cells 2 (TREM2) is a cell-surface receptor expressed by microglial cells. Individuals homozygous for rare inactivating mutations in either TREM2 or DAP12, develop a lethal form of progressive dementia known as Nasu-Hakola disease. Moreover, genome–wide association studies have shown that a mutation of TREM2 correlates with an increased risk of developing Alzheimer's disease (AD). Microglia have the crucial role to engulf and eliminate apoptotic membranes and neuronal cell debris. TREM2 receptor is involved in this process and indeed TREM2 deficiency impairs microglia phagocytic capacity. Since during brain development, microglia monitor and eliminate neuronal synapses through a process known as synaptic pruning, we evaluated TREM2 deficiency consequences on both microglial physiological functions, neuronal development and brain connectivity. Immunohistochemistry performed on brain sections and flow cytometric analysis and characterization of microglia from adult brain, revealed that TREM2-/- microglia display a more ramified appearance and a lower activation profile respect to their WT counterpart. Moreover, electrophysiological and immunohistochemical analysis performed on hippocampal slices showed an increased excitatory activity and synaptic proteins expression in TREM2-/- mice. Finally, we demonstrated both *in vitro* and in vivo by immunofluorescence and flow cytometric analysis that TREM2 -/- microglia is unable to perform a correct synaptic pruning. All these data highlight the critical role played by microglia during brain development and focus the attention on TREM2 gene as a key factor in microglia activity. Understanding the role of TREM2 at the interplay between microglia and neurons will possibly clarify some of the key mechanisms underlying neurodevelopmental and neurodegenerative diseases.

MURINE THYMIC NK CELLS ARE DISTINCT FROM ILC1S AND HAVE UNIQUE TRANSCRIPTION FACTOR REQUIREMENTS

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In the last years, many lymphoid subsets involved in the innate immune response in addition to the well-known Natural Killer (NK) cells has been discovered. Group 1 innate lymphoid cells (ILC) includes NK cells and ILC1s, a group of cells that mediate the response to intracellular pathogens. ILC1 and conventional (c)NK cells share many characteristics but they have different developmental origins.
A unique subset of NK cells has been identified in the thymus of adult mice with a NK-cell developmental pathway distinct from that in the bone marrow. This subset of thymic NK (tNK) cells shares many characteristics attributed to ILC1, like the dependence on the transcription factor GATA3, the expression of the IL-7 receptor alfa (CD127) and the absence of CD11b and Ly49 receptors, whose expression characterizes mature cNK cells. Therefore, tNK cells have been described with hybrid features of immature NK cells and ILC1, whether these cells are related to cNK cells or ILC1 has not been fully investigated.
The aim of this work was to characterize the thymic Group 1 ILCs, focusing on the transcription factor requirements and phenotypic characteristics, to provide a deeper insight into their identity and developmental relationships with other subsets of Group 1 ILCs.
We used thymus of C57Bl/6 mice lacking some genes of transcription factors known to be involved in the development and differentiation of cNK cells and ILC1, and we have analysed thymocyte phenotype and functions by flow cytometry.
We found that ILC-like population in mouse thymus is composed of two subsets: tNK cells with transcription factor requirements similar to that of cNK cells and NKT lymphocytes. In fact, tNK cells require NFIL3 and express EOMES (transcription factor essential for cNK cells), and developed independently of TBET (essential for ILC1), confirming their placement within the NK lineage. Moreover, tNK cells resemble cNK cells rather than ILC1 in their requirements for the E protein transcription factor inhibitor ID2 (which is essential for all ILCs and cNK cells), and they are largely EST1-independent even though this transcription factor, required for the correct development of cNK cells, prevented tNK cell from the acquisition of the conventional NK cell maturation markers CD11b and KLRG1.
Our data demonstrate that tNK cells have developmental requirements consistent with the NK cell lineage.

SET-UP AND VALIDATION OF CYTOFLUORIMETRIC TOOLS TO MEASURE INTRACELLULAR AND EXTRACELLULAR NAD^+ LEVEL

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Nicotinamide adenine dinucleotide (NAD^+) is a redox coenzyme and also an indispensable substrate for NAD^+ consuming enzymes involved in essential biological processes including signal transduction, gene expression, DNA repair and cell death. In order to study dynamic fluctuations of NAD^+ as well as its intracellular and extracellular distributions, we used a NAD biosensor engineered by combining a circularly permuted Venus (cpVenus) fluorescent protein with a bipartite NAD-binding domain modeled from E.coli DNA ligase, an enzyme that exclusively uses NAD^+ as substrate. Sensor fluorescence has an excitation peak at 488 nm that decreases according to NAD^+ concentration. At the same time, a second excitation peak at 405 nm is unaffected by substrate variations, serving as internal control and calibration of the system. We transfected and stably transduced the cytoplasmic sensor or its control cpVenus protein into HeLa and HEK293T cells and we performed cytofluorimetric analysis: we used ratiometric 488/405 nm values to normalize fluorescence changes to the sensor expression levels. To obtain a robust confirm of the sensor specificity, we measured fluorescence after cells treatment with a NAMPT inhibitor (FK866) able to deplete intracellular level of NAD^+ of 90%. Consistently, we observed that 16 hours of FK866 treatment is able to increase the geometric mean ratio (488/405) of cells expressing the sensor in a NAMPT inhibitor dose-dependent manner. This biosensor can now be used to dynamically follow NAD^+ modifications in live cells following different treatments. Studies are ongoing to obtain an extracellular-exposed variant of the biosensor. The strategy will be to clone the NAD-sensing construct into a specific vector containing the GPI anchor protein of human folate-receptor. This validated system offers an alternative and unexplored approach to detect both *in vitro* and *in vivo* extracellular and microenvironmental level of NAD^+.

HARMONIZING IMMUNE-MONITORING LABORATORIES OF REGIONE LAZIO: AN INTER-LABORATORY MULTICOLOR FLOW CYTOMETRY PANEL

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Determination of the immune profile of oncologic patients is essential to identify immunological biomarkers and requires standardized assays for a successful cross-study and cross-center comparison of data.
Here we present a proposal for validation, standardization and harmonization of a multicolor flow cytometry panel in a consortium-based proficiency testing involving laboratories of the immunomonitoring working group within the "Lazio Network for Translational Medicine and Cancer Biotherapies".
To this aim, we designed a polycrhomatic flow cytometric panel consisting of a mixture of dried antibodies (Duraclone, Beckman Coulter) for identification of memory/naïve T cell subsets in human peripheral blood: CD4 FITC, CCR7 PE, CD8 PeCy5.5, CD3 PeCy7, as well as a live/dead discriminator (LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, Life Technologies). Based on expression of CCR7 and CD45RA, different T cell subsets can be distinguished in naive ($CCR7^+CD45RA^+$), central memory ($CCR7^+CD45RA^-$), effector memory ($CCR7^-CD45RA^-$) and terminally differentiated ($CCR7^-CD45RA^+$).
To date, the following centers, all located in Regione Lazio, are involved in this project: Istituto Superiore di Sanità (ISS), IFO-Regina Elena, Ospedale Pediatrico Bambino Gesù, Università Cattolica, Policlinico La Sapienza, Ospedale Spallanzani.
ISS, main center of the project, will manage logistics and distribute frozen PBMCs samples, workflow, protocols and reagents to peripheral centers. Each participating laboratory will perform experiments on replicate samples and send data to the central laboratory, that will elaborate them by mean of control charts and evaluate technical variation between experimental runs.
After a successful titration and compensation test in a pilot staining experiment on PBMCs derived from a healthy donor buffy-coat, we licensed Beckman Coulter Italia to start producing the antibody mixture.
For the cross-site experiments, PBMCs were isolated from 3 healthy donor buffy coats and 60 vials (7-10 million cells/vial) of each buffy coat were frozen in liquid nitrogen. Frozen PBMCs samples as well as Duraclone tubes and SOPs will be distributed to participating centers by September 2017. After experimental processing, sites will return to ISS main center results derived from their own gating strategy as well as FCS files for a centralized analysis.
Data will be reported in a final peer reviewed consensus article around December 2017.

AUTOMATION IN THE STUDY OF LYMPHOCYTE SUBSET

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Introduction The CD4+ T-cell count is a critical parameter in monitoring HIV disease. Flow cytometry remains the gold standard technology for enumeration of CD4+ T-cells, because of its accuracy, precision and reproducibility. The AQUIOS CL is a fully automated flow cytometer with integrated sample loading, preparation and analysis. In this study the results of the T, B and NK cell lymphocyte subsets using AQUIOS Tetra method was compared to the Navios Tetra system. **Methods** The AQUIOS CL instrument is a load-and-go IVD flow cytometry system that was recently cleared by the US FDA for testing in clinical labs. The system incorporates on-board sample preparation and automated analysis with LIS capabilities. The instrument employs a volumetric approach for enumerating specific cell populations. In this study, the AQUIOS CL system performance for immunophenotyping lymphocyte cell populations was compared to the Navios tetra system , a currently used flow cytometry method for measuring the T, B and NK-cells. The whole blood samples were prepared within 24±2 hours of collection. The same specimens were prepared and analyzed by both systems in duplicates. For analysis on Navios instrument, samples were prepared manually and the red blood cells were lysed using the ImmunoPrep reagents and a TQ-prep instrument from Beckman Coulter, Inc. The statistical analysis included data from the first replicate only. Specimens: One hundred (102) specimens, including HIV+ clinical patients (70) were analyzed in the study. All testing was performed on spent blood after clinical testing had been performed. Specimens were targeted for normal and clinical range on the CD4+ T-cells. The distribution included CD4 expression levels at 32 cell:s/µL – 1500 cells/uL **Results**: In the study we found an excellent correlation between the observed and expected results with R values higher than 0.9 in all instances No significant changes were observed in the lymphocyte subpopulation results in samples stored for 48 hours at room temperature or for 72 hours at 4oC.We found an excellent correlation between of the definitive commercialized Aquios and Navios for the absolute counts of CD3+, CD3+/CD4+, CD3+/CD8+, CD3-/CD19+ and CD3-/CD56+/CD16+ cells, with R coefficients of 0.93, 0.945, 0.965, 0.945 and 0.957. **Conclusions**: Aquios overall performance is good, with excellent results in reproducibility and linearity. Results are highly correlated with those obtained with Navios. Overall Aquios it is a good instrument for the lymphocyte subs et enumeration and its simplicity of operation.

THE IMMUNOREGULATORY ROLE OF DOPAMINE ON CD8+ TREGS LYMPHOCYTES GENERATION AND SUPPRESSIVE FUNCTION

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Previous studies show that dopamine can affect the function of CD4+CD25+ Tregulatory (Treg) cells in mouse and human systems. Furthermore, it has been demon-

strated that human CD4+ Tregs constitutively express both D1 and D2 receptor classes, and contained catecholamines such as dopamine, noradrenaline and adrenaline. Catecholamine release results in the down-regulation of Treg-dependent inhibition on proliferation of CD3-activated T lymphocytes.
The aim of the project is to evaluate if dopamine impacts the generation and/or their suppressive function of non antigen-specific CD8+CD28- Tregs in vitro generated through IL-10 treatment.
In particular, our purposes has been: at first, to investigate the expression of five dopaminergic receptor subtypes, previously and after in vitro CD8+ Treg generation; secondly, evaluate the effects of dopamine on Treg generation and suppressive function, and which dopaminergic receptor is involved in.
Our results showed that precursors CD8+CD28-T lymphocytes, after IL-10 dependent regulatory commitment for seven days, can upregulate the expression of D4 dopamine receptor, with respect to CD8+ Treg precursors.
Furthermore, in vitro generation of CD8+ Tregs in presence of dopamine and D2 receptors antagonist, as Haloperidol, promotes a downregulation of their suppressive function. The involvement of D1 receptors is confirmed by the fact that D1 agonist, SKF-38393 promotes the downregulation of Tregs suppressive function.
Moreover, dopamine can downregulate the suppressive function of in vitro generated CD8+Tregs, and the involvement of D1 receptors has been demonstrated by the regulatory effect of SKF-38393 on their suppressive function.
In conclusion, the data would suggest that, like CD4+ CD25+ Treg lymphocytes, also non antigen specific CD8 Tregs can be affected by dopamine, acting through D1 like dopamine receptors.

IL-21 PROMOTES COOPERATION BETWEEN CD56DIM NK AND B CELLS

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Background. Human NK cells are characterized by NK cell receptors with inhibitory and activating function that finely control their functional activities. The majority of peripheral blood (PB) human NK cells are characterized by a $CD56^{dim}CD16^{bright}$ phenotype, whereas a minority has a $CD56^{bright}CD16^{dim}$ phenotype. CD16 is a strong activating receptor with low affinity for immunoglobulin G (IgG) able to bind immune complexes and IgG-coated, opsonized cells. Binding of this receptor with the crystallisable fragment of IgG on the opsonized target cells activates $CD56^{dim}/CD16^{bright}$ NK cells to kill through antibody dependent cell cytotoxicity (ADCC) and to produce cytokines (mainly IFN-gamma and TNF-alfa). Of note, IL-21 (a T cell cytokine) has been described to induce the switching of Ig produced by B cell to IgG1 and IgG3 isotypes, for which FcγRIIIA receptor (CD16 of NK cells) displays a higher affinity. On the other hand, we have recently described the crucial function of IL-21 in the generation of $CD56^{dim}/CD117^{neg}$ NK cells (resembling the PB $CD56^{dim}CD16^{bright}$ subset) from CD34+ hematopoietic progenitors.
Material and Methods. Purified CD34+ cells from peripheral blood buffy coats have been obtained by immunomagnetical positive selection and cultured for 15-25 days with Flt3-L, IL-15 and IL-21. Antibodies to different antigens were used to distinguish between the two NK cell subsets and to detect CD16 antigen expression.
Results. After 15 days of culture with IL-21, two subsets expressing CD56 molecule at low (CD56dim) and high ($CD56^{dim}$) density were clearly distinguishable. CD16 receptor, absent on $CD56^{dim}$ cells, was expressed at low-intermediate density of expression on the majority of $CD56^{dim}$ cells. After 25 days of culture, the $CD56^{dim}$ NK cells showed the increase of both CD56 and CD16 antigen density, indicating their further maturation and activation.
Conclusions. Through IL-21 secretion, T cells would be able to coordinate an ADCC and a specific cytokine response, inducing both an adequate isotype switching in B cells and the generation of $CD56^{dim}CD16^{bright}$ cells, an ADCC/cytokine specialized NK cell subset. The ability of IL-21 to induce CD56dimCD16bright NK cell generation should also be considered in antibody-mediated anti-tumor therapies.

EFFECTS OF IN VIVO PROTON IRRADIATION ON MOUSE T AND B CELL PHENOTYPE AND FUNCTIONS

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Ionizing radiations can impair immune functions exposing the organism to a higher risk of infections and immune-mediated disorders. The intensity and duration of the immune-compromised phase and its recovery depend on the dose, dose-rate and quality of radiation. In recent years, there has been a great interest in the effects induced by protons, both for a better assessment of the health risks associated to space missions in astronauts and for a better understanding of their effects in radiotherapy for oncologic patients. The aim of this study was to investigate the effects of the *in vivo* exposure to a proton beam (27 MeV, 2Gy integral dose) generated by the TOPIMPLART accelerator on mouse lymphoid spleen cells. During the exposure, mice were anesthetized to keep them in the right position. Sham-exposed anesthetized mice were used as controls. Each mouse was individually analyzed for several parameters (5 mice/group) 24 hours, and 1, 2 and 4 weeks after irradiation. The results showed that the number of total nucleated cells in the spleen was slightly affected by the exposure. Within lymphocytes populations, $CD4^+$ T, $CD8^+$ T and $Foxp3^+$ Treg cell numbers were reduced whereas B cell sub-populations (CD19/IgM/IgD) were only slightly affected. Spleen cells were stimulated with an anti-CD3 antibody or LPS to induce T or B cell activation, respectively. Both T and B cells were functionally impaired by the exposure. T cell proliferation was indeed reduced by 50% in exposed mice compared with controls 24 hours after irradiation. This reduction persisted up to 2 weeks after exposure and was recovered by 4 weeks. Twenty-four hours after irradiation, cytokine production (IL-2, IL-10, IL-6, IL-4, TNFα and IFNγ), analyzed by multiplex Cytometric Beads Array, was reduced in irradiated mice compared with controls. B cells also displayed a reduced cell proliferation in response to LPS (-33%) 24 hours after irradiation but their proliferative response recovered in 1 week. IgM and IgG production reduced 24 hours after exposure, further

decreased at 1 week and recovered at 4 weeks. In conclusion, in vivo local exposure to protons induced a reduction in spleen cell populations with differences according to lymphocyte subpopulations, in cytokine production, cell proliferation and antibody production. These effects persisted up to 1-2 weeks and were recovered within 4 weeks. This work was partially supported by the Italian Space Agency (ASI, BIOxTREME project) and Regione Lazio (TOPIMPLART project).

ANTI-TNF-ALPHA THERAPY INDUCES MICROBIAL AND IMMUNOLOGICAL CHANGES IN DEXTRAN SODIUM SULPHATE CHRONIC COLITIS

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Background. Anti-TNF alpha represents the best therapeutic option to induce mucosal healing and clinical remission in patients with moderate-severe ulcerative colitis. On the other side gut microbiota plays a crucial role in pathogenesis of ulcerative colitis but few information exists on how microbiota changes following anti-TNF-alpha therapy and on microbiota role in mucosal healing.

Aim. Evaluate gut microbiota and adaptive immune system response change following anti TNF-alpha therapy in murine dextransodium sulphate (DSS) colitis.

Materials&Methods. C57BL/6 mice were fed for 5 days with 3% DSS in drinking water. At day 3 of DSS treatment, mice received intravenous administration of 5 mg/Kg of infliximab (IFX), an anti-TNF alpha monoclonal antibody, or placebo. Further 2 groups of mice received IFX or placebo without DSS. Disease severity was scored daily using the four points Disease Activity Index (DAI). At day V and XII serum, colon, feces and mesenteric lymph node (MLN) were collected from each animal. Microorganisms belonging to Bacteroides, Clostridiales, Enterobacteriaceae and Fecalibacterium prausnitzii were assessed by qPCR, following bacterial DNA extraction from feces. Th1, Th2, Th17 (evaluated by intracellular cytokine staining) and Treg cell distribution in the MLN was assessed by flow cytometry.

Results. Anti- inflammatory species (Bacteroides, Clostridiales and Fecalibacterium prausnitzii) decreased during DSS-induced colitis and increased in fecal samples of IFX-treated colitis mice compared to control mice. Conversely, pro inflammatory microorganisms belonging to Enterococcaceae genera increased during colitis and decreased after IFX treatment. Furthermore, in IFX-treated colitic mice, microbial changes are associated to an initial increase (day 5 of the colitis) in Treg cells and a consequent decrease (day 12 of colitis) in Th1, Th2 and Th17 cells. Similarly, healthy mice treated with IFX showed the same histological features, microbial and immune changes of untreated colitic mice.

Conclusions. Anti-TNF alpha treatment in experimental model of colitis improves disease activity through changes in T cell subsets and in microbiota composition. Furthermore, the present study suggests that different components of the microbiota each distinctly influence the differentiation and accumulation of specific populations of immune cells. Further analysis on immune cells within mucosa will be necessary.

CHARACTERIZATION OF AN IMMUNOREGULATORY NETWORK IN MENINGIOMA PATIENTS

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Meningiomas are common intracranial tumors, displaying a benign outcome in most of the cases. However, the WHO recognizes also malignant invasive histotypes with high recurrence rate. The dual function of the immune system in the tumor microenvironment, which can act either enhancing or contrasting the tumor growth, underlies the importance of the characterization of the immune infiltrate. One of the mechanisms of the tumor-induced immune tolerance is the expansion of a heterogeneous population of myeloid-derived suppressor cells (MDSC) endowed with suppressive activity, whose presence positively correlates with tumor progression. Aim of this study is to provide a phenotypical and functional characterization of the leukocyte infiltrate in meningiomas, focusing on the MDSC subsets. Peripheral blood and freshly resected tumor tissue were obtained from 25 meningioma patients at surgery. Multiparametric flow cytometry analysis was used to provide a complete characterization of the immune infiltrate, while the suppressive activity of leukocyte subsets was tested by separating monocytes from PBMC and myeloid fractions from tumor tissue. Three circulating MDSC subsets were significantly expanded in the blood of meningioma patients in comparison to healthy donors. However, circulating myeloid cells did not show immunosuppressive activity. At the tumor site an expanded immune infiltrate was detected, mostly characterized by $CD33^+$ cells, greatly composed of immune suppressive macrophages, and variable percentages of granulocytes, both expressing the suppression-related marker PD-L1. Moreover, we observed a subset of T cells expressing the exhaustion markers LAG3 and CD279. To conclude, we found that meningioma patients display an alteration of the immune compartment, with an expansion of three circulating

MDSC subsets. At the tumor site, an immunosuppressive microenvironment is present, mostly constituted by macrophages, together with T cells expressing typical markers of exhaustion.

EFFECT OF MIRNAS DERIVED FROM *MORINGA OLEIFERA* ON PROLIFERATION, APOPTOSIS AND IMMUNOMODULATORY ACTIVITY IN TUMOR CELL LINES AND PBMCS FROM HEALTHY DONORS

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Moringa oleifera Lam. (MO) is one of the most distributed species of Moringaceae family which is also widely used in African traditional medicine. This plant has a high nutritional value for its primary metabolites and essential nutrients like vitamins, proteins and amino acids. It is also an important source of many bioactive compounds like carotenoids, polyphenols and glucosinolates.
M. oleifera have also anticancer activity, inhibiting tumour cells proliferation and inducing apoptosis.
Considering the limited data available about MO mature seeds, we evaluated by flow citometry the anti-proliferative and pro-apoptotic effects of MO mature seed aqueous extracts (MOE), in different tumor cell lines and in PBMCs from healthy donors (HD PBMCs).
We have also investigated the effects of MOE treatment in modulation of CD4+ T cells response.
Our results demonstrated that MOE reduced cell growth and induced apoptosis in human tumor cells but not in HD PBMCs. In addition, MOE treatment had immunomodulatory effects in PBMCs changing the differentiation process of CD4+ T cells in activated PBMCs, and restoring CD3+CD4+ subtype in PBMCs exposed to cyclophosphamide.
All parts of *M. oleifera* are known for their therapeutic properties: antioxidant, anti-inflammatory, antiviral, hypoglycaemic, anti-diabetic, anti atherosclerotic activities and immune boosting.
Recent works have reported that miRNAs derived from plant foods are also functional in mammals, regulating the expression of host genes and, for this reason, the effects of miRNAs extracted from MOE on human cell proliferation and death regulation were also investigated.
To evaluate the contribution of vegetal miRNAs contained in the MO extract, the pool of miRNA was purified from MO seeds and transfected in tumor cells and PBMCs from healthy donors.
Our results highlighted the ability of MO miRNA pool to reduce the proliferation and induce apoptosis in tumor cells, as also performed by total MOE. These effects were associated with a remarkable collapse of the mitochondrial membrane potential and down regulation of anti-apoptotic Bcl-2 protein.
In conclusion, MOE are able to regulate proliferation and apoptosis in different way in healthy cells respect to cancer ones and our results suggest that MO effects are due to the combination of different components, especially miRNAs.

DEFECT OF ADAPTIVE IMMUNITY IN ADA2 DEFICIENCY PATIENTS

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Introduction: ADA2 Deficiency is a new autoinflammatory disease characterized by systemic vasculopathy and episodes of strokes. However, some patients can present mild immunodeficiency. This defect is due to a loss of function mutation of CECR1 gene, coding for Adenosine Deaminase 2 protein. This protein regulates the catabolism of extracellular adenosine, converting adenosine to inosine. Much more information are available about ADA2 function in macrophages, but the knowledge about its role in adaptive immune function is limited.
Objectives: As some DADA2 patients show hypogammaglobulinemia and recurrent infections, we investigated the role of CECR1 mutation on B and T cell function.
Patients and Methods: 12 patients carrying homozygous mutations in CECR1 were examined. They showed clinical history with livedo reticularis, fever, vasculitis and neurological symptoms. We analyzed peripheral B and T cell phenotype by flow cytometry, CECR1 gene expression in B and T lymphocytes by qRT-PCR. B cells isolated from HDs and DADA2 patients have been cultured alone or in co-culture with CD4+ T cells. In vitro B cell proliferation and differentiation to Immunoglobulin secreting cells in response to TLR9 agonist have been evaluated by CFSE dilution and ELISA assay. Moreover cytokines production from T cells has been evaluated.
Results: Flow cytometric analysis showed a significant reduction in the pool of memory B cells in DADA2 patients compared with age matched controls.
Moreover we observed a significant decrease in CD4+ and CD8+ cells; interestingly an increase in pTFH cells but a significant reduction in IL21 producing cells after coculture stimulation was observed; Then we addressed B cell defect in DADA2 patients, focusing on the interaction between B and T cells. Analysis of B cell response showed that the proliferation of mutated B cells and Igs secretion were much more reduced in the presence of patient's T cells than with normal T cells.
Moreover we observed that B cells express and secrete ADA2 but ADA2 activity in patients' cells is completely abrogated.
Conclusions: Our study show that CECR1 mutation leads to an impairment in B cell function and to an altered T cell help of B cells and suggests a possible intrinsic defect in B lymphocytes.

ACTIVATION OF INNATE IMMUNE DEFENCES IN AIRWAY EPITHELIAL CELLS EXPOSED TO BACTERIAL LYSATES

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Recurrent respiratory tract infections are one of the most common reasons of disease in adults and children, particu-

larly when the immune system is deficient. The oral administration of Polyvalent Mechanical Bacterial Lysate (PMBL) is currently employed as a preventing strategies able to avoid an excessive and prolonged exposure to antibiotics. However, despite some positive outcomes of this approach, the mechanism of action of PMBL remains poorly understood, although some active immunization against PMBL components has been reported upon their administration.
Epithelial cells represent the first barrier against pathogens. They are able to sense bacterial components and activate several mechanisms of resistance, such as increase of cell adhesion molecules, secretion of autocrine growth factors and direct killing of pathogens by producing antimicrobial peptides.
We analysed by both classical and imaging flow cytometry (Image Stream) the effects of PMBL on normal human bronchial epithelial cells, showing that PMBL are able to modulate the surface expression of ICAM-1 and E-cadherin. Remarkably, we observed a polarization of epithelial cell adhesion molecule (EpCAM) to the site of cell-to-cell contact. Expression of the intranuclear marker of proliferation Ki67 increased in epithelial cells upon stimulation with PMBL, indicating a relevant proliferative effect. These data suggest a possible role of PMBL in increasing epithelial tissue tightness and thickness, which are known to play a critical role in the maintenance of epithelial barrier integrity and in tissue regeneration.
Our preliminary data underline the capability of PMBL to activate airway epithelial cells, thus indicating a possible novel mechanisms of action for PMBL in preventing the onset of infections. The awareness that airway epithelium represents an active component of immune defence and that bacterial lysate can exert an immunomodulatory effect on it, could pave the way to a more rational administration of PMBL aimed enhancing different arms of both innate and adaptive immune response against pathogens.

INNATE IMMUNITY AND VASCULAR INJURY MARKERS ARE PROGNOSTIC FOR SKIN FIBROSIS IN SYSTEMIC SCLEROSIS

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Patients affected by systemic sclerosis (SSc) show variability in clinical presentation and in disease progression. The unpredictability of disease evolution presents particular challenges for clinicians. Physicians have to decide without any reliable laboratory parameters, which patient does not need to be treated, and which is at high risk to develop more aggressive disease. Currently, the standard measure for the extent of skin disease and fibrosis is physical examination of the patient, quantified by the modified Rodnan skin score (MRSS). Unfortunately, clinical disease markers have done little to predict the trajectory of SSc. Therefore, identifying prognostic biomarkers of progressive disease is one of the highest priorities to aid clinical management in SSc patients. We have already reported that in skin biopsy mRNA expression of TLR4, MD2 and CD14, expressed mostly by macrophages/monocytes, correlates significantly with progressive skin fibrosis, as measured by the change in MRSS six months after the skin biopsy was performed (ΔMRSS). We recently have found that also endothelial cell mRNA expression markers are prognostic of worsening of skin fibrosis. In conclusion in early diffuse SSc overexpression of innate immune activation and vascular injury in the skin of SSc patients are prognostic of clinical deterioration and fibrosis.

NIVOLUMAB TREATMENT OF METASTATIC RENAL CANCER PATIENTS IMPAIRS TREGS AND POTENTIATES NK FUNCTION: THE ROLE OF CXCR4 INHIBITION (THE "REVOLUTION" TRIAL)

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Background: Despite encouraging results, in metastatic renal cancer (mRCC) Nivolumab response is not as wide as expected. The ReVoluTion clinical trial is an osservational trial aiming to identify biomarkers predictive of Nivolumab efficacy through evaluation of Tregs and NKs function. In addition, since tumoral Tregs express high level of CXCR4, antagonism to this receptor will be evaluated *in vitro* as possible inhibitor of Tregs suppressive activity. We report the preliminary results on 9 mRCC patients enrolled and evaluated up to six months of treatment.
Patients and methods: Tregs and NKs function was evaluated at day 0, 14, 28, 90, and 180 in mRCC patients undergoing to Nivolumab as second line treatment. Tregs and NKs cells were phenotypically identified as $CD4^+CD25^+$ $CD127^{low}$ $Foxp3^+$ and $CD3^-CD56^+CD107a^+$, respectively. Tregs activity was evaluated through the soppression of T effector proliferation and NKs activity through K562 dependent cell cytotoxicity. The effect of CXCR4 antagonism was evaluated ex vivo on Tregs and NKs activity.
Results: At this time 9 patients were evaluable: 2 died of disease (DOD), 2 progressed at six months (PD6), 3 presented partial response at 3 months (PR3) and 2 patients showed stable disease at three months (SD3). In 2 patients initial T effector anergy was revealed but recovered during Nivolumab treatment. In 4/9 patients the PD/DOD corresponded to increase in Tregs activity while in 3/9 patients with a PR3 increase in Teff proliferation, compatible with a decreased Tregs activity, and ex vivo Tregs inhibition through CXCR4 antagonists was detected. Though the total peripheral number of Tregs was unaffected, a significant decrease in the suppressive Tregs was revealed. Suppressive Tregs (high PD1 and CXCR4) at time 6 months displayed a reduction in the expression of PD-1 and CXCR4 during Nivolumab treatment ($p<0.001$ and $p<0.05$, respectively). NK function increased during Nivolumab treatment with a concomitant reduction in the expression of the inhibitory receptors CD158a ($p=0.02$), PD-1 ($p=0.04$) and CXCR4 ($p=0.24$). In the 4/9 patients that experienced PD/DOD the higher Tregs function accompanied a reduction in NK cytotoxicity.
Conclusion: Nivolumab treatment in mRCC patients determined detectable variations on Tregs and NKs function. Tregs suppressive function was impaired by inhibition of CXCR4 receptor suggesting that CXCR4 antagonism reverted Tregs suppressive activity.

DISSECTION OF CYTOKINE PROFILE AND PHENOTYPE OF CELLS INFILTRATING THE INTESTINAL MUCOSA OF CHILDREN WITH POTENTIAL OR OVERT COELIAC DISEASE BY MULTIPARAMETRIC FLOW CYTOMETRY

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BACKGROUND/AIMS: Coeliac disease (CD) is characterized by a variable combination of gluten-dependent symptoms, genetic factors, specific antibodies and enteropathy. Most patients show a variable degree of enteropathy (overt CD), but a minority shows absence of villous atrophy despite the presence of CD-specific autoantibodies (potential CD). We investigated the cytokine profile and the phenotype of intestinal T cells from children affected by overt versus potential CD.

METHODS: Cell phenotype (on unstimulated cells) and cytokine production patterns (by intracytoplasmic staining after PMA/Ionomycin stimulation) were analysed by multiparametric flow cytometry, in both gluten-raised T cell lines (TCLs) and freshly isolated mucosal cells. Jejunal biopsies were obtained from 19 overt CD (mean age 5.1 yrs), 16 potential CD patients (8.5 yrs) and 12 non-CD children (4.2 yrs). Statistical analysis was performed using a paired Student t-test ($p< 0.05$).

RESULTS: An increased number of CD3+ TCR γδ+ cells, mainly CD4CD8 double negative cells, was found in TCLs from overt CD patients compared to potential CD ($p<0.004$) subjects. A higher fraction of IL-4 producing cells, mainly CD4+ cells, was detected in TCLs from children with potential CD ($p<0.0007$). Ex vivo analysis on freshly isolated intestinal cells confirmed the significant increased frequency of TCR γδ+ cells in gut mucosa of children with villous atrophy ($p<0.02$). However, a higher percentage of TCR γδ+ cells was detected in potential CD compared to healthy mucosa of non-CD controls ($p<0.04$). An increased expansion of IL-4 producing CD4+ T cells was found in biopsies from potential CD compared to overt CD patients ($p<0.05$).

CONCLUSIONS: Our study confirms in CD patients an expansion of TCR γδ+ T cells, particularly in subjects with enteropathy (overt CD). The transition to villous atrophy seems to be characterized by a dramatic disappearance of IL-4 producing cells. These findings may offer biomarkers useful to characterize the different stages of CD.

Oncology

CHARACTERIZATION OF THE IMMUNOMODULATORY PROPERTIES OF MESENCHYMAL STEM CELLS ISOLATED FROM WHARTON'S JELLY

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Background: Extraembryonic tissues such as umbilical cord are considered a promising source of stem cells, potentially useful in therapy. The characterization of cells from the umbilical cord matrix (Wharton's Jelly) and amniotic membrane revealed the presence of a population of mesenchymallike cells, sharing a set of core markers expressed by "mesenchymal stem cells".

Aim of the study: We aimed to evaluate the global phenotype and immunoregulatory molecules expression in undifferentiated WJ-MSC cultured at different passages. The evaluation of immunomodulatory potential of WJ-MSC was performed through MLR (Mixed Lymphocyte Reaction), one-way or two-way, by incubating cells with PBMCs or enriched lymphocytes and quantitative assessment of lymphocyte proliferation performed after incorporation of CFSE.

Materials and methods: Freshly isolated WJ-MSC were cultured in standard media. Flow cytometry was used to characterize cells at 2nd, 5th, 10th passage. Further characterization was made by immunocytochemistry analysis. To perform MLR, cells were incubated with PBMC or enriched lymphocytes previously loaded with CFSE, in order to track their proliferation using flow cytometry. After 5 days of incubation, marked PBMC were counted and data plotted and analyzed. Positive and negative controls were run simultaneously.

Results: WJ-MSC did express the classical MSC markers at all the tested passages. Moreover they resulted highly positive for class I MHC and negative for class II ones. MLR was performed at different MSC/lymphocyte ratios, showing a global trend which demonstrated that MSC are able to reduce proliferation of responder cells in MLR. In particular, ratios of 1:1 and 1:2 cells: lymphocytes showed results which reached the statistical significance.

Conclusion and future objectives: After this initial characterization we have shown that cells extracted from Wharton's jelly are adherent to the MSC phenotype. in addition, we characterized the expression of a number of novel molecules by these cells, some of which are known for their immunomodulatory activity. As a result, WJ-MSC were able to inhibit lymphocyte proliferation, both mitogen-induced or to allogeneic cells. These data confirm the great interest on the phenotype of these cells, for which growing roles are proposed in regenerative medici-

ne. Further data will be needed to evaluate the expression of immunomodulatory molecules in the differentiated WJ-MSC, and to evaluate the inhibitory potential of differentiated cells in MLR reactions *in vitro*. Characterization of these properties is mandatory for the subsequent creation of specific preclinical models of cell therapy.

IOMM-LEE AND CH-157 MENINGIOMA CELLS: IN VITRO ASSESSMENT OF SURVIVAL AND RADIATION RESPONSE

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Meningiomas are a heterogeneous group of tumors and they account for 30% of all cancers of the Central Nervous System. These tumors are treated surgically, but in some cases radiotherapy (RT) is also administered. Experimental investigations on the biology of meningiomas and the biological basis of the RT action did not have wide application due to the scarcity of in vitro and in vivo models. Recently some improvement has been achieved thanks to cell cultures. Based on literature data, we used the immortalized cell lines derived from aggressive variants of the tumor, the IOMM-Lee and the CH-157. So, the aim of this study is to evaluate the response to the radiation of these meningioma cells. They were irradiated in single fraction with increasing doses from 2 Gy to 15 Gy using two different linear accelerators, Synergy® (SYN) and Cyber Knife® (CK). They have a different dose rate: 3 Gy/min and 10 Gy/min respectively. We obtained the survival curves accordingly to the data of clonogenic tests. Then were carried out the study of apoptosis and cell proliferation, with Annexin V – PI and MTS respectively. Our results showed a similar dose-response of both meningioma cell lines, with increasing cell death at increasing doses. Moreover, the IOMM-Lee showed a more radiation resistance than the CH-157. Indeed, the cellular death of CH-157 was obtained at a very low dose irradiation. For this reason, we conducted following investigations using IOMM-Lee cell line only. Another element seems to have an important role in radiation response: the dose-rate delivery. In fact, with the higher dose-rate the response to radiation was greater than that obtained with the lower one. Furthermore, in our experiments the necrosis has had a role less important than apoptosis: the rate of apoptotic cells is greater than that of the necrotic cells at any dose of irradiation and at any time of analysis. So, we can underline the need to investigate more specifically the pathways associated with apoptosis and DNA damage repair. These findings need to be confirmed and validated by further studies with a greater number of samples and with experiments focused not only on cell lines, but also on primary cultures. In conclusion, we can confirm a dose-response effect due to the early and late apoptosis.

ADVANCED GOLD NANOPARTICLES FOR BREAST CANCER TREATMENT: SYNTHESIS, CHARACTERIZATION AND IN VITRO BIOLOGICAL INVESTIGATION

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Breast cancer is still a relevant problem in human health. Various nanostructures-based drug delivery systems have been synthesized to improve the therapeutic selectivity. Among them, gold nanoparticles (AuNPs) are the most notable owing to their unique characteristics that make them elective agents for the conjugation with drugs and for photothermal therapy. However, the potential toxicity of AuNPs remains a major hurdle that impedes their use in clinical settings. The aim of the study was to develop a stable nanosystem composed by a gold core, a biocompatible polymeric nanoparticle (e.g. poly(amido)amines (PAA)s or PLGA) and an anticancer molecule. The polymeric coating might not only reduce the cytotoxicity of AuNPs but might also facilitate the loading and delivery of drugs. Stable colloidal AuNPs with diameters in the range 4 - 20 nm were synthetized and investigated. The colloidal stability was monitored with x-ray diffraction, ultraviolet-visible spectroscopy and dynamic light scattering. Sequentially, AuNPs with or without polymeric coating were conjugated with Herceptin, a chemotherapic agent against HER positive breast cancer cells. Their efficacy was evaluated *in vitro* using two breast cancer cell lines (SKBR-3, EGF Receptor HER2 overexpressing and MCF-7) and fibroblasts cells (NIH3T3, negative control). Cell morphology and cell viability were evaluated by MTT assay, Confocal Laser Scanning Microscopy, and Scanning Electron Microscopy analysis. Our results showed a toxicity effect of AuNPs against SKBR3 cell line, whilst MCF7 and NIH-3T3 were not affected. Moreover, polymer coated-AuNPs functionalized with Herceptin exerted higher toxicity in comparison with free drug and uncoated AuNPs, consistent with the endocytosis capability of the nanoparticles in the target cancer cells. Although we are still performing studies to confirm the efficiency of this strategy, we suggest that the newly formed biocompatible nanosystems may represent a suitable platform for the conjugation of many types of antineoplastic molecules.

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EARLY HISTOLOGICAL CHANGES INDUCED BY BORON NEUTRON CAPTURE THERAPY ON NORMAL AND NEOPLASTIC RAT LUNG

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Lung cancer represents one of the leading cause of death in western world. Multiple lung metastases or spread tumours, such as malignant pleural mesothelioma, still lack a valid treatment strategy. Boron Neutron Capture Therapy (BNCT), highly selective and able to destroy isolated cancer cells while sparing normal ones, should represent an alternative or an adjuvant option to standard therapies. BNCT is based on the killing effect of high-LET alfa particles originated from the nuclear capture reaction occurring when ^{10}B atoms are exposed to neutron irradiation. High neoplastic to normal tissues boron concentration rate is essential to minimize damages to the lung, being one of the most radiosensitive organs. The present study, performed in vivo on groups of healthy and neoplastic rats exposed to neutron irradiation with and without boron administration, aims at evaluating the extent of radiation induced damages by histological analysis, in order to assess the feasibility and the efficacy of lung/BNCT. As a first step the early radiation effects on healthy lung and on neoplastic nodules were evaluated, analysing biopsy specimens of animals sacrificed at fixed times within two months post-irradiation. Histological analyses of healthy tissues did not evidence signs that typically characterize the acute phase of radiation damage. Only a moderate inflammation and slight signs of fibrosis were shown at the longer observation times, thus suggesting that minimal collateral damages were induced to surrounding normal lung tissue. Conversely evidence of severe radiation damages, such as increased cell size, spread necrotic areas and fibrosis were present in metastatic nodules of BNCT treated animals, confirming the therapeutic potential of the treatment.

CINNAMOMUM ZEYLANICUM AS PRO-OXIDANT AGENT IN MELANOMA CELLS

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Since survival of melanoma patients remain poor (5-year survival below 25%), there is continued need for new therapeutic approaches. Many studies have shown that plants are a potential source of natural molecules than can be used as agent in cancer treatment. Cinnamon is a small and evergreen plant belonging to Lauraceae family, native to Asia, whose medical properties are still under investigation. In particular, *Cinnamomum zeylanicum* besides anti-microbial, anti-mycotic and anti-inflammatory properties, have been demonstrated to have an antitumor effect. In this study we investigated the anti-melanoma cell activity of *C. zeylanicum* essential oil on an in vitro established melanoma cell line. The results revealed that this agent has an antiproliferative effect as evaluated by colony forming assay and cell cycle analysis. We also found that reactive oxygen species (ROS) production was augmented by the treatment with *C. zeylanicum* essential oil indicating its pro-oxidant effect in melanoma cells. In addition, *C. zeylanicum* essential oil was able to improve the effect of a conventional antitumor drug such as Tamoxifen used at low doses. An interesting indication of the possible mechanism involved in the observed effects is the *C. zeylanicum* essential oil capacity to regulate the melanoma Labile Cell Iron (LCI) amount and to modulate a group of Iron metabolism genes. Our results strongly support the potential use of this plant in combination cancer therapy.

MESENCHYMAL STEM CELLS (MSCs) AND AML CELLS DO NOT SHARE IDENTICAL CHROMOSOMAL DEFECTS: A CYTOGENETICS, FISH AND aCGH/SNPa STUDY

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Concerns have been raised on the possibility that MSCs may undergo malignant transformation and various studies have discovered cytogenetic defects in MSCs from AML patients (pts) suggesting that they may represent a peculiar mechanism of leukemogenesis.
We tested whether leukemic cells and MSCs from 18 AML pts share the same cytogenetic defects on conventional cytogenetics (CC), FISH and aCGH/SNPa. Bone marrow cells from all the 18 pts were obtained at clinical diagnosis after informed consent and submitted to CC, FISH and aCGH/SNPa. Mononucleated cells were isolated from BM by density gradient centrifugation and culture flasks at a cell density of 10^6 cells/cm^2 at 37°C, 5% CO2 in MEM-alpha medium containing 1% Penicillin/Streptomycin, 1% L-Glutamine and 10% fetal bovine serum. MSCs were examined after the first passage and their phenotype was evaluated by flow cytometry. The following FISH commercial probes were applied according to manufacturer's guidelines: LSI D7S486/CEP7, LSI AMLETO from Abbot Molecular Inc. (Chicago, Il, USA) and ON c-Myc/SE8, SE10(D10Z1) from Kreatech (Amsterdam, NL). aCGH/SNPa was carried out with the SureScan Microarray Scanner G4900DA (Agilent Technologies Inc. Santa Clara, CA). CC on AML cells revealed a normal chromosomal pattern in 10 pts, a -7 in 2 pts, a del(7)(q31) in 1 pt, a +8 in 2 pts, a +10 in 1 pt, a t(8;21)(q24,q22) in 1 pt and a complex karyotype in the last pt. All these defects were confirmed by FISH. On FISH examination the MSCs from all the 18 pts showed a normal pattern. In contrast, on aCGH/SNPa the MSCs from 6 pts presented chromosomal alterations. An amplification of the entire chr. 5 was discovered in 1pt,(FISH showed a true +5) a LOH of a 3.8 Mb sized region located on 13q31.1 in 1pt, a LOH of a 4.3 Mb region mapped on chrs 6 and 18 in 2pts, an amplification of three 71Kb, 322Kb and 47Kb sized regions of chrs 5, 18 and 20 in 1 pt and an amplification of three 17Kb, 40Kb and 70Kb sized regions of chrs 9, 11 and 15 in another pt who presented an amplification of six distinct genes including *JAK1, ELN, FGFR2*. In conclusion i) MSCs from chromosomally abnormal AMLs may have a normal FISH pat-

tern, but on aCGH/SNPa may contain LOH or amplifications different from those of leukemic cells; ii) the MSCs defects may flag a leukemogenic-induced genomic instability which affects not only the hematopoietic tissue but also the niche; iii) aCGH/SNPa is a useful technique to identify potential clonal markers.

EVALUATION OF FIBROBLASTS RELEASED FACTOR INFLUENCE ON CANCER CELL INVASION AND MIGRATION EXPOSED TO LOW AND HIGH LET RADIATION

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Understanding the mechanisms of local recurrences or metastases that occur after radiotherapy represents a therapeutic challenges. Many reports described that photon radiation may increase the migration and invasiveness of cancer cells surviving after radiotherapy whereas the few studies with carbon ions performed so far showed a different modulation.
In this study, we evaluated the influence of the microenvironment on cancer cell invasion analyzing the effects of untreated fibroblasts released factors on irradiated pancreatic cancer cells using different doses (0.5Gy, 1Gy, 2Gy and 4Gy) of photons and carbon ions.
Our results indicated that photons seemed to slightly increase the invasiveness of pancreatic cancer cells when co-cultured with normal fibroblasts whereas carbon ions radiation appeared to induce a response in fibroblasts resulting in inhibition of cancer cells invasion.
In particular, we observed an increase in invasiveness following photon radiation more evident with 1 Gy. Differently, high-LET radiation decreased the amount of invading cells already with 1 Gy, remaining steady after 2 Gy and 4 Gy.
The changes in migration and invasiveness we observed with the of normal fibroblasts, are probably due to the reciprocal release of soluble factors by cancer cells and normal fibroblasts whose production is differently modulated after high or low LET radiation. These results point out how important it is to consider the role played by tumour microenvironment when effects of radiation are evaluated.

DIETARY SUPPLEMENT BASED ON GANODERMA LUCIDUM TESTED ON HUMAN GLIOBLASTOMA CELL CULTURE

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We tested the effect of a supplement containing both phyto and mycotherapic components and made by Miconet, an Academic spin-off of the University of Pavia. The phyto-mycotherapic supplement, "Ganostile" product by Miconet s.r.l. contains Ganoderma lucidum (also known as reishi), Eleutherococcus senticosus, Echinacea purpurea, Astragalus membranaceus.
In the traditional Chinese medicine, the mushroom Ganoderma lucidum has long been used in the treatment of many diseases including cancer. Antitumor activity of extracts from medicinal mushrooms could be due at least in part by fungal beta-glucan component. We tested in vitro the effects of the supplement using U251, a human glioblastoma multiforme cell line that is the most malignant of all brain tumours. The aim of this study was to use the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS) assay to determine the response of cell culture to different concentrations of phyto-mycotherapic supplement. After several time of continuous exposure treatment, U251 cell vitality drastically decreased compared to the control. This data was also confirmed by cytofluorimetric analysis by which, at the same concentration, we found the presence of apoptotic cells. In MTS assay at low concentrations the cell vitality appears to increase, but by cytoflurimetric analysis we have found that most of cells were blocked in a specific phase of cellular cycle and these results could suggest a preliminary effect of the supplement on the cell cycle. To arrest cells in a particular phase could be a good strategy to take cancer cells in a more susceptible stage, in which the treatment with new chemioterapic compounds acts specifically in this cell cycle phase could exert the maximum effect. The possible cytotoxic effect of natural compounds against cancer cells may help future antitumor development against poorly differentiated cancer cell subpopulations.

DENDRITIC CELLS SUBSETS PROFILES IN CLASSICAL HODGKIN LYMPHOMA AT DIAGNOSIS AND UPON TREATMENT COMPLETION: ANALYSIS ON 54 PATIENTS

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Background: Up to one-third of classical Hodgkin lymphoma (cHL) pts recur upon induction treatment, while those cured may suffer from troublesome iatrogenic sequelae. While efficacy/toxicity tradeoffs relying on clinical prognosticators remain unsatisfactory, prospectively validated and reproducible biomarkers are lacking. The relevance of the inflammatory microenvironment in cHL prompted us to investigate the role of the key immunomodulators myeloid type-1 (mDC1), type-2 (mDC2) and plasmacytoid (pDC) dendritic cells (DCs). **Methods**: We assessed blood DCs levels in 54 newly diagnosed pts and 29 age-matched healthy controls through multiparametric flow cytometry. The Blood Dendritic Cell Enumeration Kit from Miltenyi Biotec (Bergisch-Gladbach, Germany) was used, with 0.27 (r,0.09-0.42), 0.02 (r,0-0.04), 0.19 (r,0.09-0.37) as reference median

rates for mDC1, mDC2 and pDC, respectively. Mann-Whitney, Wilcoxon sum rank, and log-rank two-sided tests were used as appropriate with p<0.05 regarded as significant. **Results**: The median age was 34 years (r,19-72); advanced stage (IIB extranodal or bulky, III, IV), bulky disease and B-symptoms affected 57%, 44% and 46% of pts. All but two pts received ABVD regimen. The relative and absolute counts of all DCs subsets were lower in cHL pts than in healthy controls (p<0.001). The median rates were inferior for the advanced vs early stage, for both mDC1 (0.032 vs 0.070; p=0.008) and mDC2 (0.005 vs 0.009; p=0.001) subsets. Also, the median mDC2 counts were reduced in case of bulky (0.004 vs 0.008; p=0.001) and extranodal (0.005 vs 0.006; p=0.019) disease. Pts with B symptoms had lower levels for mDC1s (p=0.029), mDC2s (p=0.005) and pDCs (p=0.020). At the end of treatment (EOT) 27 pts were reassessed: median (25th;75th) fractions of mDC1, mDC2 and pDC subsets raised from 0.036 to 0.167 (0.086-0.250)(p< 0.001), from 0.005 to 0.012 (0.007-0.020)(p< 0.001), from 0.042 to 0.190 (0.110 –0.300)(p< 0.001), respectively. The increases were 4.6 fold for mDC1, 2.4 for mDC2, 4.5 for pDC and aligned DCs subsets with the reference frequencies and the interquartile ranges of the controls. At a median f.u. of 35 mo.s, PFS for the 31 pts with advanced stage showed a trend in favor of those with mDC2 levels above the median (100 vs 73%; p=0.06). **Conclusions**: mDC1, mDC2, and pDC blood levels were all significantly reduced in cHL pts at baseline and fully recovered at EOT. Notably, the reduction of mDC2 counts paralleled the anatomical extent of disease, correlating with advanced as well as extranodal and bulky disease.

THE ANTI-APOPTOTIC BAG3 PROTEIN IS INVOLVED IN BRAF INHIBITOR RESISTANCE IN MELANOMA CELLS

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BAG3 protein, a member of BAG family of co-chaperones, has a pro-survival role in several tumour types. BAG3 anti-apoptotic properties rely on its characteristic to bind several intracellular partners, thereby modulating crucial events such as apoptosis, differentiation, cell motility, and autophagy. In human melanomas, BAG3 positivity is correlated with the aggressiveness of the tumour cells and can sustain IKK-γ levels, allowing a sustained activation of NF-κB. Furthermore, BAG3 is able to modulate BRAFV600E levels and activity in thyroid carcinomas. BRAFV600E is the most frequent mutation detected in malignant melanomas and is targeted by Vemurafenib, a specific inhibitor found to be effective in the treatment of advanced melanoma. However, patients with BRAF-mutated melanoma may result insensitive ab initio or, mostly, develop acquired resistance to the treatment with this molecule.
Here we show that BAG3 down-modulation interferes with BRAF levels in melanoma cells and sensitizes them to Vemurafenib treatment. Furthermore, the down-modulation of BAG3 protein in an in vitro model of acquired resistance to Vemurafenib can induce sensitization to the BRAFV600E specific inhibition by interfering with BRAF pathway through reduction of ERK phosphorylation. The molecular interactions between BAG3 and mutated BRAF may represent a target for novel multi-drugs treatment design.

NON-IONIZING RADIATION EFFECTS ON HUMAN GLIOBLASTOMA U-251 CELL LINE AND HUMAN PRIMARY FIBROBLASTS

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Glioblastoma Multiforme (GBM) is the most aggressive human brain tumour. Surgery and adjuvant radiotherapy (RT) and/or chemotherapy (CT) are the standard therapies, but new therapeutic options and a deeper understanding of how the therapies applied modulate tumor cells are needed since patients develop relapse. Several studies showed that the interaction of electromagnetic waves, especially low intensity laser, on cell culture could either stimulate, or inhibit, proliferation, depending on radiometric parameters. Biological mechanisms involved in photobiomodulation are not well defined in literature, as well as the interaction of laser's electric field. This study aims to investigate and compare the possible interaction of low intensity laser and constant electric field on tumour cell line and primary human fibroblast cell line. In this study U-251 cell line derived from a malignant glioblastoma tumour by explant technique, whereas human fibroblast derived from biopsy. The irradiation was performed with continuous red laser and with continuous infrared laser. Each treatment was conducted with two different wavelenght (660nm and 808nm) and several different power levels (R: 100-50-25 mW; IR: 120-60-30 mW), but at the same energy (4 Joule). To verify cell survival and proliferation, mitochondrial activity analysis (MTT) and Trypan blue assay were performed at 24h and 48h after treatments. Later, other experiments were carried out with cells treated with the most efficient power and different levels of energy. In order to evaluate the effect of electric field, an experiment was also carried out to verify the action of the constant electric field (direct current) on the migration of these cells. Cells were seeded and after 72h a cell-free area was created using a ferrule (Wound Assay). Immediately after, the cells were subjected to constant parallel electric field and to constant perpendicular electric field. Then cells were coloured with May-Grunwald Giemsa staining after 6h and 9h from the treatment to evaluate cell migration. Our results suggest that electric field modulates cell migration and laser could reduce mitochondrial activity of cells compared to unexposed control cells.

EXTRACELLULAR MIRNAS AS POTENTIAL BIOMARKERS IN HUMAN GLIOBLASTOMA

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Glioblastoma Multiforme (GBM) currently has no diagnostic markers or effective treatment, and the development of minimally invasive diagnostic methods is a major challenge. Extracellular microRNAs are de-regulated in most tumor types, reflecting the pathophysiological state of the primary affected tissue. Their relative stability, accessibility and efficient detection in body fluids suggest their use as "liquid biopsy"-based biomarkers of tumor diagnosis, progression and response to therapy. Recently, different subsets of circulating miRNAs have been described in cancer patient blood, and miRNAs packed into tumor-released nanosized extracellular vesicles (EVs) have been shown to contribute to tumor establishment and metastatic spread, suggesting not only diagnostic/prognostic but also therapeutic potential.
In order to identify miRNAs to be used as biomarkers for GBM, we analyzed the composition of extracellular circulating miRNAs secreted by human glioblastoma multiforme (GBM) cells, both in vitro and in a GBM orthotopic animal model. Thus, extracellular miRNAs have been studied in the EVs purified from culture cell supernatants and from the plasma of mice bearing GBM tumors. The isolated nanosized EVs have been characterized by FACS analysis, for the presence of surface EV specific markers such as CD81, CD9, and by TEM. A small group of extracellular miRNAs released by GBM cells in the plasma of GBM bearing mice has been identified by next-generation sequencing, defining a miRNA specific signature which could be potentially used as GBM biomarker and treatment-response predictor.

^{18}F-FET,^{18}F-FCH AND 18F-DOPA UPTAKE IN HUMAN GLIOBLASTOMA T98G CELLS

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Differential diagnosis between brain tumor recurrence and chemo-radionecrosis is frequently difficult despite the enormous improvement of diagnostic modalities as CT, MRI and PET.
O-(2-[^{18}F]fluoroethyl)-l-tyrosine (^{18}F-FET) is a radiolabelled modified natural aminoacid which accumulates in glioblastoma cells and ^{18}F-dihydroxyphenylalanine (^{18}F-DOPA) is an analogue of L-DOPA, used to image the dopaminergic pathway and to evaluate striatal dopaminergic presynaptic function but also gliomas. ^{18}F-methyl-choline (^{18}F-FCH) is a main substrate of cell membrane metabolism and is commonly used in molecular imaging. Aim of this study was to evaluate ^{18}F-FCH, ^{18}F-FET and ^{18}F-DOPA uptake by human glioblastoma T98G cells in basal, irradiation and bystander conditions. Cells were irradiated by photons or carbon ions at doses of 2-10-20 Gy. Bystander cells were incubated with irradiated conditioned medium harvested from irradiated cells. Controls were not treated with irradiation. Cells were incubated with equimolar amounts of radiopharmaceuticals for different incubation times. A significant uptake of ^{18}F-FCH was seen at 60, 90 and 120 minutes. The percentage uptake of ^{18}F-FET in comparison to 18F-FCH was lower by a factor of more than 3, with different kinetic curves. ^{18}F-FET showed a more rapid initial uptake up to 40 minutes and 18F-FCH showed a progressive rise reaching a maximum after 90 minutes.
^{18}F-DOPA showed the lowest uptake in T98G cells, less than a half in comparison to FET. Uptake kinetic was characterized by an early maximal activity at 40 minutes, thereafter tending to a plateau pattern. After irradiation a paradox effect was observed with FCH and FET, higher uptake for increased doses of radiation treatment, due to the upregulation of cells attempts to repair nonlethal damage. No appreciable bystander effect, mediated by putative cytokines from irradiated cells media ,was observed on FCH and FET uptake.
^{18}F-FCH, ^{18}F-FET and ^{18}F-DOPA are candidates for neuro-oncological PET imaging: ^{18}F-FET could be the most appropriate oncological PET marker in the presence of reparative changes after therapy, where the higher affinity of ^{18}F-FCH to inflammatory cells makes it more difficult to discriminate between tumour persistence and non-neoplastic changes. The lowest uptake of ^{18}F-DOPA in tumor cell line together with a physiological accumulation in basal ganglia might indicate a less suitable candidate for in vivo imaging.

POSTNATAL DEVELOPMENT OF CEREBELLUM IN "DAL" MICE: ALTERATIONS IN REGULATION OF INTRACELLULAR CALCIUM AND IN NEUROTRANSMISSION

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Prolidase deficiency is a rare autosomal recessive disease caused by mutations in the PEPD gene. The pathology is characterized by the formation of ulcers in the limbs, facial dimorphism and mental retardation. The prolidase enzyme is capable of breaking down dipeptides with proline or hydroxypoline residues at the C-terminal end of the aminoacid chain and it is involved in the catabolism of proteins. We decided to evaluate possible alterations in neurotransmission, first considering variations in $Ca2^{+}$ cytosolic presence, in mice at 10 and 60 days after birth. Indications of possible changes in intracellular calcium were obtained through the immunocytochemical expression of the $Ca2^{+}$ buffer proteins parvalbumin, calretinine and calbindine, and on PMCA1, a $Ca2^{+}$ ATPase pump. It was then attempted to ascertain whether variations in the expression of these proteins could correlate with alterations in neurotransmission circuit by evaluating some of the most important glutammatergic and GABAergic markers in the cerebellum, such as glutamate receptors

GluR2 and GluRδ2 as well as the GAD67 and GABAα6 enzyme. As for the Purkinje cells of mice from dal/+ and from dal/dal on, the results showed that not all Purkinje cells have a normal development and are distributed on a monolayer; abnormalities were observed for dendritic shaft and axon emergency cone. In addition, alterations in the proliferation and maturation of granular cells have been reported. Markers for buffer proteins have found that there is an alteration in the $Ca2^{+}$ homeostasis that may be correlated the abnormalities of GABA glutamate marker markers or GABA markers. As for other neuronal types, such as granular cells, alterations in proliferation and maturation have been reported, probably related to defects in the organization of the basal platelet membrane following reduction or absence of prolidase. Morphological and functional alterations of the development of the cerebellar cortex shown here require further studies of behavioral and electrophysiological aspects.

EFFECTS OF THE NEW CISPLATIN-BASED PT(IV)ACPOA PRODRUG IN CNS TUMOUR CELL LINE

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Neuroblastoma and glioma are the most common tumours of CNS and they are associated with a poor prognosis. For the diffuse invasion of tumour cells, surgery cannot completely remove them, for this reason adjunctive radiotherapy and chemotherapy are essential.
From many years, cisplatin has been used for systemic cancer treatment, but despite its effectiveness to induce cancer cell dead, onset of severe side effects (nephrotoxicity, ototoxicity and neurotoxicity) and drug resistance limits its clinical use.
Therefore, one of the main experimental goal in medicinal chemistry is to synthetize new platinum-based prodrug, for example platinum (IV) prodrugs, which are characterized by low effective dose, hence high cytotoxicity and less unwanted effects.
The new prodrug Pt(IV)AcPOA represents an interesting example of this approach. It generates, upon reduction in tumor cell, the cytotoxic cisplatin and 2-(2-propynyl)octanoic acid (POA), a histone-deacetylase inhibitor (eHDCAi). Likely, its higher activity is due to the fact that the inhibition of histone deacetylase leads to increased exposure of the nuclear DNA, thereby permitting higher levels of platination by cisplatin and therefore promoting killing of cancer cells.
This contribution aims to describe the morphological and functional changes during apoptosis induced in neuronal (B50) and glial (C6) rat cell lines by Pt(IV)AcPOA after a continuous exposure of 48h. The results obtained show that cell treatment with this new prodrug induces activation of different apoptotic pathways, which have been investigated by citofluorimetry, imunocytochemical detection and quantification analysis by western blotting of the protein involved.

MINIMAL RESIDUAL DISEASE AND LOG–REDUCTION OF PLASMA CELLS EXPLAIN THE SUPERIOR VALUE OF DOUBLE AUTOLOGOUS STEM CELL TRANSPLANT IN YOUNGER PATIENTS WITH MULTIPLE MYELOMA

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Minimal residual disease (MRD) by flow cytometry (FC) is a surrogate marker for survival in multiple myeloma(MM). In particular, previous studies have demonstrated that the presence of MRD at day 100 following autologous stem cell transplant (ASCT) predicts for both progression free survival (PFS) and overall survival (OS). Anyway, median PFS after single ASCT is 27 and 16 monthes within MRD negative and MRD positive patients. These results suggested that further treatments are desirable after single transplant. In support of this argument, 10 years ago it was demonstrated that double ASCT effected superior RFS and EFS than single ASCT.Anyway, majority of clinical groups have followed to treat MM with a single ASCT. Thus, we used FC to assess MRD and Log-reduction of plasma cells (PCs) to evaluate the better degree of tumor reduction after double ASCT and to investigate the best time point to predict the outcome. Bone marrow samples from 30 patients who underwent double ASCT were assessed by FC at different time points: post induction (MRD1 and LOG1), post first-(MRD2 and LOG2) and post- second (MRD3 and LOG3)ASCT. MRD (>0.01%) was evaluated by a six-color FC. Log –reduction was calculated as a logaritmic ratio between the PCs at presentation and PCs at each time of assessment. A significant difference was evidenced among the three time points from ANOVA test for both LOG-reduction ($p<0.001$) and MRD ($p=0.005$). In particular, LOG3 was significantly greater than LOG2 ($p<0.001$) and LOG1($p<0.001$). Similarly, MRD achieved after double ASCT was deeper than MRD achieved after single ASCT ($p=0.005$) and after induction($p<0.001$). Then, frequency of MRD positive patients after double ASCT was significantly lower than that found after the first ASCT (n= 15 vs n= 23,$p=0.008$) and after induction (n=15 vs n=27, $p=0.004$). When the survival analysis was considered, a significant reduction of PFS was observed in patients belonging to an unfavorable cytogenetics risk group ($p<0.001$) and patients showing a MRD over 0.01% (34.1 vs 17.6 mths, $p=0.031$) as well as a Log-reduction lower than 2.57 ($p=0.01$)after double ASCT . Results of MRD and LOG –reduction post double ASCT were confirmed at multivariate analysis ($p=0.004$ and $p=0.01$, respectively). In conclusion, our results by FC sustained the double ASCT as the goal treatment strategy in MM because of a deeper reduction of PCs, a higher frequency of MRD positive patients and a longer PFS compared to single ASCT.

IMMUNOPHENOTYPIC HETEROGENEITY OF LEUKEMIA STEM CELLS-ENRICHED SUBSETS BY MULTIPARAMETER FLOW CYTOMETRY IN HUMAN T-CELL LEUKEMIA

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T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) is a T-cell malignancy that affects children and adults. It is curable in about 80% of pediatric patients, but only 40% of adults survive over 5 years.

Refractory cases and relapses in both patients are presumably due to ineffective targeting of leukemia stem cells (LSCs), most likely resistant to standard chemotherapy. Using an integrated fluorescent reporter of Wnt signaling (known as 7TGC) containing 7 Tcf/Lef-binding sites upstream of a minimal promoter and GFP marker, we have previously reported that an active Wnt/β-catenin signaling is a defining feature of LSCs in mouse and human T-cell leukemias.

Here, we show that the Wnt-active and LSC-enriched subsets of human T-ALLs are phenotypically heterogeneous by a multiparameter flow cytometry approach. In particular, two patient derived xenograft (PDX) leukemias were transduced with the 7TGC Wnt-reporter lentiviral construct and then transplanted into immunodeficient (NSG) mice. Hence, we determined the LSC frequency of GFP+ (Wnt active) and GFP− (Wnt inactive) fractions of PDX leukemias by a limiting dilution assay and confirmed that the Wnt-active leukemic subsets were enriched with LSCs. In parallel, the 7TGCtransduced human leukemia cells were also assessed with 3 flow cytometry panels, each containing up to 8 fluorophore-conjugated antibodies against extracellular markers, including CD7, CD1a, and CD34, described as associated with LSCs. Flow data were analyzed using the t-SNE algorithm to generate a

dimensional reduction of the 10 flow cytometric parameters (8 markers plus FSC and SSC) to 2 t-SNE dimensions and to describe the intratumoral heterogeneity within the cell populations. The t-SNE analysis of GFP+ (Wnt active) cells showed that some subpopulations overlap with each other and variably express specific surface markers, highlighting the relatedness of two human leukemias. Of note, expression heatmaps of CD7 and CD1a markers showed distinct CD1a-CD7+ subpopulations within Wnt-active cells, raising the possibility that only few subpopulations within the Wnt-active subsets might show LSC activity.

Taken together, these preliminary observations support the idea that novel information can be derived from highly dimensional analysis by flow cytometry with important implications for the understanding of tumor heterogeneity in human T-ALLs and the dynamics of Wnt-active leukemic populations in response to conventional cytotoxic chemotherapies.

MULTIPARAMETER FLOW CYTOMETRY FOR MINIMAL RESIDUAL DISEASE ANALYSIS IN NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS: RESULTS FROM THE ITALIAN PATIENTS UNDERGOING EMN02/HO95 PHASE 3 TRIAL

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Multiple Myeloma (MM) is an incurable disease in which complete response (CR) can be reached after therapy. Multiparameter Flow Citometry (MFC) is a highly sensitive method to measure the depth of response by evaluating Minimal Residual Disease (MRD) in the bone marrow (BM). BM immunophenotype was performed at diagnosis in 718 italian MM patients undergoing EMN02/HOVON clinical trial; MRD sub-study involved 214 young MM patients, who reached at least VGPR. Treatment consisted of Bortezomib-Cyclophosphamide-Dexamethasone (VCD) induction, mobilization and stem cell collection, intensification with Bortezomib-Melphalan-Prednisone (VMP) vs one or two High-Dose-Melphalan (HDM) followed by stem cells transplant, consolidation with Bortezomib-Lenalidomide-Dexamethasone (VRD) vs no consolidation, followed by Lenalidomide maintenance. MRD analysis was performed after intensification/consolidation in the pre-maintenance phase, after 6 courses of maintenance, and thereafter every 6 months until progression. A double antibody combination (CD138Fitc/CD20PerCp-Cy5.5/CD117APC/CD45APC-H7/CD38PE-Cy7;
cyKappaFitc/cyLambdaPE/CD19PerCp-Cy5.5/CD56APC/CD45APC-H7/CD38 PE-Cy7) was used: the first tube was employed to obtain PCs quantification, the other one to validate PCs clonality. MRD-negativity was reached when <20 clonal PCs were detected among ≥2.000.000 leukocytes (<0.001%), with a sensitivity of 10^{-5}. Two hundred-fourteen Italian patients (118 male/96 female) with a median age of 57 years (35-65) entered MRD sub-study. Fifteen (15%) pts were ISS stage III at diagnosis. 134/214 (63%) had received HDM and 79/214 (37%) VMP. At pre-maintenance phase a total of 168 patients were evaluated for MRD analysis. 122/168 (72.6%) patients were MRD-negative: 78/105 (74.3%) in the HDM vs 44/63 (70%) in the VMP group (p = 0.506). After a median follow-up of 30 months the 3-year PFS was 61% in MRD-positive vs 74% in MRD-negative patients (HR 3.66, 95% CI: 1.24 – 10.84; p<0.001). Finally, 31% of MRD-positive patients at pre-maintenance who had a second evaluation after at least 1 year of lenalidomide maintenance, became MRD-negative. MRD by MFC is a strong prognostic factor in MM and a feasible technique to detect residual tumor cells among patients reaching at least a VGPR. Our preliminary results show that, in patients reaching at least VGPR, maintenance therapy further improved depth response in both arms.

TRAP1 CONTROLS CELL CYCLE G2-M TRANSITION THROUGH THE REGULATION OF CDK1 AND MAD2 EXPRESSION/UBIQUITINATION

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Regulation of tumor cell proliferation by molecular chaperones is still a complex issue. Here, the role of the HSP90 molecular chaperone TRAP1 in cell cycle regulation was investigated in a wide range of human breast, colorectal and lung carcinoma cell lines and tumor specimens. TRAP1 modulates the expression and/or the ubiquitination of key cell cycle regulators through a dual mechanism: i) a transcriptional regulation of *CDK1*, *CYCLIN B1* and *MAD2*, as suggested by a gene expression profiling of TRAP1-silenced breast carcinoma cells, and ii) a post-transcriptional quality control of CDK1 and MAD2, being the ubiquitination of these two proteins enhanced upon TRAP1 downregulation. Mechanistically, TRAP1 quality control on CDK1 is crucial for its regulation of mitotic entry, since TRAP1 interacts with CDK1 and prevents CDK1 ubiquitination in cooperation with the proteasome regulatory particle, TBP7, this representing the limiting factor in TRAP1 regulation of the G2-M transition. Indeed, TRAP1 silencing results in enhanced CDK1 ubiquitination, lack of nuclear translocation of CDK1/Cyclin B1 complex and increased MAD2 degradation, whereas CDK1 forced upregulation partially rescues low Cyclin B1 and MAD2 levels and G2-M transit in a TRAP1-poor background. Consistently, the CDK1 inhibitor, RO-3306 is less active in a TRAP1-high background. Finally, a significant correlation was observed between TRAP1 and Ki67, CDK1 and/or MAD2 expression in breast, colorectal and lung human tumor specimens. This study represents the first evidence that TRAP1 is relevant in the control of the complex machinery that governs cell cycle progression and mitotic entry and provides a strong rationale to candidate TRAP1 as a biomarker to select tumors with deregulated cell cycle progression and, thus, likely poorly responsive to novel cell cycle inhibitors.

Key Words: TRAP1, cell cycle, CDK1, MAD2, mitotic entry.

FLOW CYTOMETRY IN THE NEW CANCER IMMUNOTHERAPY ERA

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Over the past 30 years, the applications of flow cytometry (FCM) in clinical oncology started with the DNA content analysis for both ploidy and proliferative activity analysis as potential prognostic parameters in solid tumors. The availability of monoclonal antibodies and the development of multicolor flow cytometers widely enlarged the spectrum of the clinical applications of FCM that became a fundamental tool for the diagnosis and prognostic definition as well as for defining minimal residual disease after treatment in haematological diseases. In clinical immunology, FCM is a fundamental analytical platform for assessing and monitoring the immunophenotype of HIV-positive patients, for classifying various immune deficiencies and for studying the patient immune reconstitution following allogeneic bone marrow transplantation. Among the emerging clinical applications in the field of the study of metastatic spread in solid tumors, the quantification of endothelial progenitor cells as well as the identification and characterization of circulating tumor cells are very promising. The recent introduction of several effective approaches of anticancer immunotherapy for solid tumors, that has been defined the cancer immunotherapy revolution, strongly accelerates the interest on the definition of the immunological profile of cancer patients before and during treatment, the identification and monitoring of immunological targets and the validation of new predictive/prognostic biomarkers with analytical methodologies to be applied in clinical setting. After implementation of both methodological (i.e. timing of analysis during treatment, sample collection and storage) and clinico-biological (i.e. selection and validation of reliable immune response biomarkers) variables, FCM-based techniques could effectively become an ideal tool for the optimization of new immunotherapeutic approaches in cancer patients.

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