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Italiana di
Citometria

PROCEEDINGS OF THE XXXI NATIONAL CONFERENCE OF CYTOMETRY

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Lucca, Italy
October 8-11, 2013

EDITED BY
R. DE VITA and G. MAZZINI

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XXXI National Conference of the Italian Society of Cytometry GIC
October 8-11, 2013
Lucca, Italy

The XXXI Italian Society of Cytometry (GIC) bi-annual meeting had been held in Lucca (Tuscany, Italy) from October 8th to 11th. 2013.
All abstracts were carefully reviewed by the Scientific Program Committee and published here in full and categorized by scientific tracks such as cell cycle and apoptosis; environmental sciences and toxicology, hematology, immunology, methodology-technology, and oncology.
To date there are over 600 members actively involved in educational programs, promotion of quality controls programs, drafting/validation of guidelines and accreditation, providing information for people involved that actively work in the field of basic and applied cytometry.

This years the Conference had been preceded by a half a day satellite event devoted to the establishment of the "Professional Certification GIC Register" which will be committed to test, train, certify and monitor the professional levels of the "Italian Cytometrists" under the responsibility of GIC. At present more than 50 members have applied to be enrolled in the formative training structure. After the series of "dedicated seminars" the attendees had the opportunity to discuss with the "Entry Committee" the consistency of the basic requirements they have declared in a preliminary "entry form". Among all the CV items applicants should have a minimum of three years practical expertise in the field. Anyone passing this entry level will be examined by a formal "Examination Committee" that formally assignee the title of "Qualified Cytometrist". This new GIC initiative had been very much appreciated by the members.
Regarding the scientific program this year the Scientific Committee selected 31 abstracts for oral presentation among those submitted (110). Plenary sessions included 9 invited lectures focused on the contribute of cytometry in different application fields, highlighting the interdisciplinary profile of GIC. The opening lecture was given by Prof. Dario Campana (University of Singapore) and was focused on how flow cytometry can guide treatment decisions in acute leukemia. This outstanding talk offered a rational and fascinating overview on the role of flow cytometry in the diagnosis and clinical management of acute leukemias including the future perspectives of monitoring the patent's individual response to therapy in modern clinical trials.
Each Parallel session, offered along three days, was tailored on the traditional topics such as immunology, oncology, hematology, methodology, and environmental sciences with cytometry as the common denominator. Several invited and selected lectures were given in order to bring the discussion on the more emerging and promising aspects of flow cytometry.

Again in the "core" of the Society activities a special session had been programmed to the reports of the ongoing GIC Projects:

a) "Professional Qualification": R. De Vita summarized the strategic importance in Italy (as everywhere in the world) to apply a "formal-serious-system" to take care and guarantee the high Professional level of people (Beginners as well as Seniors) involved in the area of analytical cytometry especially those having clinical impacts. The "national-governance" should have in mind the patients-care and the scientific society may have a crucial role in these initiatives.
b) "The role of Flow Cytometry in the immunophenotyping of Acute Leukemia." G. Gaipa made an exhaustive details of what the board of this Project did in the recent past and what the enrol-

led “Expert Committee” is doing in the evaluation of the selected literature in order to be able to define in the future step of the activity witch is the real impact of Flow Cytometry in this very important and promising area of application of this technology.

c) “Standardization of a Flow Cytometry approach for the evaluation of Endothelial Circulating cells and their progenitors”. M. Della Porta reported the activities of the group of labs (spread around the north Italy) practically involved in the analyses of reference samples prepared in Pavia and rapidly sent to the participants. The very ambitious Original Design involving a very large number of markers did results in interlaboratories CVs of the preliminary samples extremely wide. In a successive step and thanks to the “analytical-instrumental-setting” pre-defined by the Committee the actual output of the trial is going to be greatly improved. The support of the companies (Beckman Coulter and Miltenyi Biotec) had been extensively acknowledged while the cost of the Project was out of the financial possibility of the GIC Society.

d) “Quality assessment of the cytometric Laboratory”. R. Chianese made a summary of the activity she has devoted first to introduction and spread around the members of the Society the concept of the “Quality” in the cytometry labs and therefore to the way to improve and maintain the minimum level. The preliminary phase of this initiative have enrolled only few labs but thanks to the capillary sensibilization action (carried out with the support of the GIC secretary office) the situation had been significantly improved and there is now a large group of users all around Italy interested to be trained in quality up-grade. Very important will be the forecast interactions between this Project and the one dealing with “Professional Qualification” while the “Quality items” will be certainly one of the mile stones to be upgraded in the professional culture of the next new generation of cytometrists.

The Conference had also been characterized by two special symposium (topics were “Mimimal residual disease” and “Circulating tumor cells”), in which selected experts from the main Italian Societies in the fields of Hematology and Oncology has brought their top contribution.
Poster session included 79 contribution and a special committee have selected 2 Poster award winner in each category.
A substantial contribution has been provided by the principal companies in the field which had been located in a large exhibition area inside the conference center.
The President conclusive remarks included the great satisfaction for the very good scientific level of all the sessions with particular reference to the “Posters” definitely established as the most common way to introduce novel data and discoveries in science.

President	Past President
G. Gaipa	F. Lanza

EDITED BY
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Invited Speakers

FLOW CYTOMETRY TO GUIDE TREATMENT DECISIONS IN ACUTE LEUKEMIA

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In addition to its established application to the diagnosis of hematologic malignancies, flow cytometry can contribute in many meaningful ways to patient clinical management. For example, leukemia subtypes that are associated with a poor response to therapy can be identified based on their cell marker profile. Early T-cell precursor acute lymphoblastic leukemia (ETP-ALL) is one such type, characterized by a defined immunophenotype. Another area where flow cytometry can help providing valuable prognostic information is in the detection of minimal residual disease (MRD), i.e., leukemic cells undetectable by standard morphological methods. The most reliable approaches to study MRD in acute leukemia are flow cytometric detection of aberrant immunophenotypes and polymerase chain reaction amplification of rearranged immunoglobulin and T-cell receptor genes and chromosomal breakpoints. These methods can detect 1 leukemic cell among 10,000 or more normal bone marrow or peripheral blood cells. The results obtained with the two methods in childhood ALL are highly concordant. In patients with acute myeloid leukemia (AML), flow cytometry is the only method that allows MRD monitoring in the majority of patients. Prospective studies of MRD in patients with newly diagnosed ALL and AML have shown that presence of MRD detected by flow cytometry in bone marrow is strongly and independently associated with a higher risk of relapse. There is strong evidence that MRD levels before hematopoietic stem cell transplantation in patients with ALL are closely related to the risk of relapse post-transplant; emerging data indicates that this correlation also occurs in AML. The discovery of new markers to distinguish leukemic from normal cells should improve the reliability and sensitivity of MRD studies by flow cytometry, and further widen its application.

PROBING THE BRAIN BY LIGHT: MEASURING INTRACELLULAR PH AND CHLORIDE CONCENTRATION BY MEANS OF *IN VIVO* TWO-PHOTON SPECTROSCOPY

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The correct operation of the brain is assured by the fine tuning of excitation and inhibition between neurons. A crucial determinant of the strength of inhibition is the intracellular concentration of Chloride [Cl]i, with low levels of $[Cl]_i$ in the postsynaptic neuron indicating a strong inhibition. It is becoming clear that disruption in Cl homeostasis is implicated in a great varieties of brain dysfunctions, but, up to now, no suitable tool has been available to measure $[Cl]_i$ in the intact brain. ClopHensor is a ratiometric non-FRET biosensor based on a highly Cl- and pH-sensitive GFP variant (E^2GFP), fused with a red fluorescent protein, which fluorescence is independent on pH and [Cl-]. In this study our aim is to use this sensor in the anesthetized animal by means of 2-photon *in vivo* spectroscopy. In order to do this, we have to: a) understand the 2-photon excitation properties of the indicator and b) determine an imaging protocol that allows the independent ratiometric estimates of pH and Cl-. Two-photon spectroscopy of the recombinant sensor and of cultured cells is used to build a calibration model that allow to compute pH and $[Cl]_i$ from the observed spectra. However, the transport of these calibration data to the *in vivo* imaging is not straightforward: the brain is not transparent and both absorption and scattering of light are strongly wavelength-dependent. Therefore the spectral signature of the sensor is strongly distorted by the tissue overlying the measured cells. Here, we will present a procedure that allow to compute the spectral distortion of the sensor caused by the brain tissue. In this way we can estimate absolute $[Cl_i$ and pH in vivo in the intact brain in normal and pathological conditions. By using two mode locked laser, it is possible to perform dynamic measures of Cl fluxes during brain operation, thus opening a new window on our understanding of neuronal inhibition.

CHROMOSOME GENOMICS: A QUANTUM JUMP IN ANALYZING COMPLEX PLANT GENOMES

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Plants exhibit large variation in genome size and a number of them, including wheat have genomes many times larger than that of human and comprising many billions base pairs. Genomes expanded mainly due to repetitive DNA and polyploidy. DNA sequence redundancy hampers gene mapping and cloning as well as genome sequence assembly. An attractive approach to overcome this difficulty is to dissect genomes to small parts and analyze them separately. Chromosomes are natural units of nuclear genomes and mitotic metaphase chromosomes can be sorted by flow cytometry in a number of plants. The sorted chromosomes have been used to simplify physical genome mapping using PCR and FISH, develop DNA markers from particular chromosomes and construct BAC libraries. The availability of chromosome BAC libraries facilitates construction of ready to sequence physical maps of complex genomes, and their production has underpinned the international effort to sequence the wheat genome. The spread of next generation sequencing methods opened new horizons for chromosome genomics. Chromosome survey sequencing is a powerful way to study molecular composition of individual chromosomes, identify majority of genes, develop virtual linear gene order models and improve whole genome sequence assemblies. Chromosome sorting was initially limited by inability to discriminate chromosomes with the same DNA amount. One solution is to use cytogenetic stocks in which chromosome size has been changed due to translocations and/or deletions. Moreover, a careful selection of sort windows enables to obtain fractions highly enriched for chromosomes of interest. A more refined approach relies on labeling specific chromosomes prior to flow cytometry using fluorescence in situ

hybridization in suspension (FISHIS). Thus, chromosomes can be sorted from various materials and chromosome genomics has been increasingly used to facilitate the analysis of large and polyploid genomes where whole genome approaches may fail.

SILICON OPTICAL MICROCHIP FOR NEW CYTOMETRIC APPROACHES IN CELL BIOLOGY

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Investigations of cell cultures in 3D microenvironments have attracted an increasing interest by research scientists in the biomedical area. Although cells typically reside in vivo in a three-dimensional (3-D) environment, most of what is known about cells has been derived from cultures performed on flat surfaces, such as plastic Petri dishes or glass slides. Therefore, there is an increasing interest in investigating cell cultures on 3-D matrices, also known as scaffolds, since these structures can have major effects on cell behavior with regard to adhesion proliferation, differentiation and, also, apoptosis.
We have recently proposed the use of silicon devices based on a well-ordered material as a three-dimensional supporting matrix for biological nanostructures and for optofluidic applications. A cell-selective silicon microincubator, that incorporates a vertical, high aspect-ratio (HAR) silicon photonic crystal (PhC) as core element, has been successfully demonstrated for performing cell cultures in a 3-D microenvironment[1] [2]. HAR PhCs consist of periodic arrays of parallel ≈ 3 μm-thick silicon walls separated by ≈ 5 μm-wide, 50 μm-deep air gaps, fabricated by electrochemical micromachining (ECM) of <100>-oriented silicon wafers. PhCs are artificial materials characterized by the presence of photonic bandgaps, i.e., wavelength intervals in which the propagation of the electromagnetic field inside the material, in our case in direction orthogonal to the silicon walls, is prohibited. The definition of interaction effects between PhCs and cell cultures represents a first significant step toward the fabrication of a new-concept cell-based optical biosensor, in which cells grow and proliferate into HAR PhC transducers, for direct label-free optical monitoring of cellular activities.
As a first step we have investigated the peculiar behaviour of different cell lines belonging to two different embryonic origin such as the epithelial versus the mesenchimal. The experimental results strongly support our statement that only cells with mesenchymal phenotype posses the peculiar ability to actively colonize the gaps, adherent to the inner vertical surfaces of the silicon walls, thus demonstrating that these HAR micromachined structures, as microincubators, exhibit a sort of cell selectivity. These findings support our idea to use silicon PhC as both cell-selective microincubator and optical “labell-free” transducer of cell morphology for advanced biomedical applications [3]. Variation in cell morphology as well as adhesion/detachement to silicon walls are easily probed by the system acting as a core point of a family of lab-on-chip respectively dedicated to the EMT (probing morphology) and to apoptosis (probing cell detachement). Applications in the fields of tumor biology as well as in the drug-discovery will be described.
Very promising are the implication of cell biomechanics and transforming potential (cancer mechanobiology) being our silicon PhC a suitable device to study the different ability of cancer cell to penetrate the narrow microcavity according to their biological aggressivity. Preliminary experiments on tumour cell lines derived from a variety of tumors of various grade of malignancy have confirmed that those deriving from high grade are to enter the PhC microcavity much more than those from low grade.
Final step of our future researchers is again in the area of clinical oncology: the search and evaluation of the “Circulating Tumor Cells” in the peripheral blood of tumor patients seem to be very promising to be performed with our technology. CTC are in fact characterized by peculiar “bio-mechanical properties” to have the ability to strech inside the microcapillary, where they undergo the EMT process and finally they posses (by definition) the high biological aggressivity to be able to originate in a distant site the metastasis of the original tumor. Work partially supported by Fondazione Cariplo, grant no. 2011-0308.

PERIPHERAL BLOOD DENDRITIC CELLS IN HUMAN HEALTHY AND PATHOLOGICAL PREGNANCY

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Pregnancy provides a major challenge to the maternal immune system, which must tolerate fetal alloantigens encoded by paternal genes for allowing the fetus to grow and develop in the uterus despite being recognized. Dendritic cells (DCs) are widely distributed potent antigen-presenting cells that bridge the arms of innate and adaptive immunity. They can either promote or prevent immune activation, thus playing a central role in the control of immune tolerance. DCs in pregnant women have been characterized mainly at the maternal-decidual interface, where their state of activation has emerged as one of the key players influencing the feto-maternal immunological equilibrium. Growing evidences indicate that during pregnancy also DCs circulating in the peripheral blood, which is the most accessible source of DCs, undergo changes that may be relevant to the adaptation of maternal immune responses needed to allow fetal growth. Moreover, alterations of circulating DCs that may likely reflect alterations occurring in the decidua have also been described in pathological pregnancies, such as in pregnancies complicated by intrauterine growth restriction or pre-eclampsia.

RECEPTOR-MEDIATED REGULATION OF HUMAN NK CELL HOMING RECEPTOR-MEDIATED REGULATION OF HUMAN NK CELL HOMING

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After recruitment in peripheral tissues by chemokine gradients, NK cells must undergo a priming process in order to acquire full functional competence before migrating towards lymph nodes. Priming takes place when NK cells interact with other innate immunity cell types, either resident or recruited during inflammation (which release a set of relevant cytokines) as well as by the recognition of virus-infected or tumor target cells.
In this context, the interaction of NK cells with Dendritic cell (DC) during the early phases after pathogen invasion appears to play a crucial role in the generation of down-

stream adaptive T cell immunity and in the acquisition of particular chemokine receptor involved in the homing process, such as CCR7. We showed that freshly isolated peripheral blood KIR$^+$ NK cells, as well as KIR$^+$ NK cell clones, *de novo* express CCR7 upon co-culture with mature DC. As a consequence, they become capable of migrating in response to the CCR7-specific chemokines CCL19 and/or CCL21. This mechanism is tightly regulated by KIR-mediated recognition of HLA class-I. Analysis of NK cell clones revealed that KIR-mismatched but not KIR-matched NK cells acquire CCR7. Moreover, we showed that KIR2DS1$^+$ NK cells upon interaction with HLA-C2$^+$ target cells acquire CCR7 and migrating properties. These data have important implications in haploidentical haematopoietic stem cell transplantation (HSCT), in which KIR-mismatched NK cells may acquire the ability to migrate to lymph nodes, where they can kill recipient's antigen presenting cells (APCs) and T cells thus preventing graft vs host (and host vs graft) reactions.

COMPARATIVE ANALYSIS OF FLOW CYTOMETRY AND HYSTOLOGY FOR THE EVALUATION OF LYMPHONODE BIOPSIES

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Flow cytometry (FC), is a qualitative and quantitative analysis technology; it characterizes cell populations at a single cell level. FC is considered a powerful tool for the diagnosis of mature B-cell lymphoid neoplasms and may assist in the diagnosis and classification of mature T- and NK-cell lymphoid tumors. Although FC is able to provide objective and quantitative results, even on very small samples, it is not routinely applied in the evaluation of solid tissues. In this study, we evaluated the contribution of flow cytometric immunophenotyping along with cell block immunocytochemistry as adjunct technique in the diagnosis and classification of lymphoproliferative disorders (mainly Non-Hodgkin and Hodgkin Lymphoma, as per the current WHO classification). The main aim of our study was to classify the lymphomas as accurately as possible based on FC assessment by immunophenotyping. Our data showed that FL on tissue samples is a powerful adjunct to morphology and immunohistochemistry in the diagnosis of several haematological malignancies and may provide rapid and accurate diagnosis of lymphomas.

FLOW CYTOMETRY IN THE CANCER RESEARCH LABORATORY

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In a post-genomic era, in which epigenetics is one of the major player and the gene is put in its living context, rather than detached from its material matrix, the study of the cell in its whole becomes even more actual. In particular, in the field of cancer research this approach appears to be more appropriate for the understanding of such a complex genetic diseases, thus allowing to get insight into some obscure aspects of cancer pathogenesis such as cancer cell dormancy, chemo-resistance, local and distant relapses. Tumors are known to be characterized by lack of cellular homogeneity originating from a number of sources, including differential nutrient status due to the *de novo* microcirculations of tumors, to infiltration of normal cells into the tumor, and to the hierarchical natures of the cell populations from which cancers arise, the so called tumor initiating cells (TIC). TIC are often derived from tissue stem cells and these *cancer stem cells* are characterized by resistance to most cytotoxic treatments and by a high metastatic rate.
In this scenario flow cytometry is recognized as an invaluable technology to study the complexity of cancer disease. In recent years, major advances in single-cell measurement systems have included the introduction of high-throughput versions of traditional flow cytometry that are now capable of measuring intracellular network activity and, thanks to the emergence of a variety of isotope labels, can enable the tracking of a great variety of cell markers and components. Owing to its multiparametric nature flow cytometry can provide highly detailed information on any single cell in a heterogeneous population, thus facilitating our capacity to catalog and bring order to the inherent diversity present in cancer cell populations. In addition, the wide applications provided by flow cytometry have the potential to reveal new insights into cancer biology at the intersections of stem cell function, TIC and multilineage tumor development. Its versatility and flexibility means it can be conducted in the preclinical and clinical setting, remaining at the forefront of both routine and exploratory cancer studies.

CD39 AND IMMUNOREGULATION: NOT ONLY REGULATORY CD4+ T CELLS

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CD39 is an ectoenzyme which mediates immunosuppressive functions catalyzing ATP degradation. It is not known whether CD39 is implicated in the activity of CD8+ regulatory T lymphocytes (Treg). To this issue, we analyzed CD39 expression and regulatory function in both CD8+ and CD4+CD25^{hi} Treg in two diametrically opposed clinical settings, as autoimmunity and tumor immune escape. In particular, the study evaluated peripheral Treg cells in fibrotic autoimmune disease and multiple sclerosis (MS); whereas in tumor context, CD39 expression and function was analyzed in peripheral blood as well as in tumor specimens from cancer patients. The results showed defects in the CD8 + Treg subset combined or not with abnormalities regarding the CD4 + CD25 + Treg cells in autoimmune diseases. Moreover, although CD39 was found expressed by both CD8+ (from the majority of healthy donors and tumor patients) and CD4+CD25^{hi} Treg, CD39 expression correlated only with suppression activity mediated by CD8+ Treg. Importantly, CD39 counteraction remarkably inhibited the suppression activity of CD8+ Treg (both from peripheral blood and tumor microenvironment) indicating that CD39-mediated inhibition constitutes a prevalent hallmark of their function. Collectively, these findings, unveiling a new mechanism of action for CD8+ Treg, provide new knowledge and new therapy target on both immune-mediated diseases and intratumoral molecular pathways related to tumor, immune escape.

CELL COMMUNICATION AND APOPTOSIS IN LYMPHOCYTE CELLS

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Communication with the environment is the basic principle of any biological system, with the increasing complexity of higher organisms cell had to evolve several diverse mechanisms to exchange informations crucial for tissue organization and maintenance of the organism as a whole. To date, different mechanisms for the exchange of molecular information between cells have documented: the exchange of membrane fragments (trogocytosis), formation of tunneling nanotubes (TNTs) and release of microvesicles (MVs). In our study, paring up flow cytometric and confocal microscopy approaches, we have investigated apoptosis linked to the intercellular communication pathway, in an early time-frame of stimulation. Jurkat cells and CD4+ T lymphocytes have been stained with different dyes, specific for cell membrane (PKH26and/or 67), cytoplasm (CFSE) and endo-lysosomal compartment (DiI) and stimulated with different apoptotic trigger, such as FasL, Trail and staurosporine, able to trigger them towards an extrinsic or intrinsic pathway, respectively. Our results show that the triggers differently promote cell to cell communication. Firstly, the two cell types show a different ability to form cell conjugates, an important prerequisite for cell communication. Cell communication FasL-promoted mainly proceeds along cytosolic material exchange *via* TNTs and MVs. The behaviour of Trail is similar to that one from FasL, even if the exchange is strongly reduced. On the contrary, staurosporine promotes in Jurkat cells mainly the exchange of membrane fragments. In order to further elucidate the mechanism underlying this phenomenon we have evaluated the possible involvement of calcium, using specific chelators such as EDTA, EGTA and BAPTA-AM. Taken together, our results suggest that the new routes of cell communication finely described in different biological condition also occur during apoptosis, suggesting a key role in both life and death if the cell.

MINIMAL RESIDUAL DISEASE DETERMINATION AS A SURROGATE END-POINT IN ACUTE MYELOID LEUKEMIA

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Cytogenetics and molecular abnormalities as assessed at diagnosis represent the most powerful prognosticators in acute myeloid leukemia (AML). However, since the outcome of patients allocated in the currently recognized cytogenetic/genetic risk groups is highly variable, novel prognostic factors which take into account therapy and response related features are needed. Minimal residual disease (MRD) might be one these new factors since it potentially recapitulates all of the prognosticators involved in AML response to treatment such as cytogenetics/genetics, multidrug resistance phenotype, pharmacokinetic resistance and other still unknown factors. A number of study, using flow cytometry or molecular biology, have consistently demonstrated that MRD, when present at any time-point after chemotherapy and stem cell transplantation, is a significantly independent prognostic factors for survival estimates and duration of complete remission. Based on this, MRD detection is firmly rising to the attention of the experts who envisage some unique implications potentially derived from its assessment: personalizing patient's risk-assignment and therapy, offering a more modern and realistic definition for complete remission and introducing a surrogate endpoint for outcome of trials testing new agents in AML. Especially the potential role of MRD as a surrogate endpoint is capturing the attention of the authorities such as the Food & Drug Administration (FDA). In March 2013 at the FDA headquarter, it was held a workshop focusing right on MRD detection as a surrogate end-point for clinical trials exploring new drugs in AML. A surrogate endpoint is defined as a biomarker intended to substitute for a clinical efficacy endpoint; surrogate end-points are expected to predict clinical benefit (or lack of benefit). Since MRD detection defines an end-point which occurs prior to standard end-points such as overall survival or disease free survival, it has the properties to serve as a surrogate end-point. In this view, the next challenge will be to implement the procedures for MRD biomarker qualification in a way that correlation of MRD with traditional end-points is strongly and convincingly established.

THE PARADIGM OF CHRONIC MYELOID LEUKEMIA: THE CRITICAL ROLE OF MRD MONITORING FOR THERAPEUTIC SUCCESS (AND CURE..?)

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Chronic myeloid leukemia (CML) is invariably associated with the t(9;22) chromosomal translocation. This results in a BCR–ABL fusion gene encoding a tyrosine kinase whose deregulation is both necessary and sufficient for the initiation and maintenance of the disease. CML is one of the first human malignancies for which the dream of targeted therapy has come true with the advent of imatinib, the first tyrosine kinase inhibitor (TKI), in 2001. Until then, the deeper level of response patients could achieve with interferon alpha (in no more than 20% of the cases) was the complete cytogenetic response (CCyR), defined as the disappearance of metaphases positive for the t(9;22) translocation as assessed by chromosome banding analysis. Imatinib was shown to induce CCyR in more than 80% of patients. It soon became evident that with imatinib, we could afford asking for more – that is, asking for molecular responses. Molecular monitoring tools allowing to qualitatevely detect the presence or absence of the BCR-ABL fusion transcript were already available – they had been developed in an attempt to identify those CML patients who did not require further treatment after allogeneic stem cell transplantation. Real-time reverse transcription (RT)-polymerase chain reaction (PCR) allowing to follow quantitatively the dynamics of BCR-ABL transcripts in blood and bone marrow, was then introduced in 1999. So, it was really a happy coincidence that when imatinib entered clinical evaluation, this new and powerful tool had just been made available and could thus complement the hematologic and cytogenetic follow-up of patients. Its high sensitivity soon made it the gold standard to monitor the response of patients receiving TKI therapy. The application of real-time RT PCR indeed excitingly showed that, as soon as CCyR is achieved, the leukemic burden continues to decline progressively over

time. As a consequence, two further steps beyond complete cytogenetic response were defined - Major Molecular Response and Complete Molecular Response. Major Molecular Response (MMR) indicates a three log reduction in BCR-ABL transcript level with respect to the standardized 100% baseline (hence, MMR=0.1% or lower). In a proportion of patients, however, we can observe such a reduction in residual disease burden that BCR-ABL becomes undetectable even by real-time RT PCR. This is what we call 'CMR' (Complete Molecular Response; at least four log reduction i.e. 0.01% or lower). The achievement of specific molecular response milestones at specific timepoints has now entered the definition of 'optimal response', 'warning' and 'failure'. An optimal response is defined by BCR-ABL transcript levels ≤10% at 3 months, ≤1% at 6 months, ≤0.1% at 12 months and ≤0.01% at 18 months of therapy. Second-generation TKIs (dasatinib, nilotinib) are now also available. More potent than imatinib, they are capable to induce higher MMR and CMR rates. The achievement of a stable CMR is the *conditio-sine-qua-non* for attempting treatment discontinuation. This possibility, very attractive also from a pharmacoeconomical point of view (TKIs are extremely expensive drugs), is now being explored in the context of clinical trials whose preliminary results show its feasibility at least in a proportion of patients. Supported by Fondazione CARISBO, PRIN 2009 (prot. 2009JSMKY), AIL, AIRC.

MINIMAL RESIDUAL DISEASE STUDIES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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Minimal Residual Disease (MRD) is a powerful and independent predictor of the overall response to treatment in Childhood Acute Lymphoblastic Leukaemia (ALL). The most reliable and validated methods to assess MRD in ALL are flow cytometric (FCM) analysis of leukemia-associated immunophenotypes and polymerase chain reaction (PCR) amplification of antigen-receptor gene rearrangements. However, a strong association between MRD and risk of relapse in childhood has been largely demonstrated, irrespective of the methodology used. MRD is now used in several clinical trials for risk assignment and to guide clinical management. Low levels of MRD at early time points may suggest to decrease treatment burden in patients who are likely to be cured with reduced intensity regimens. By contrast, high MRD levels at late time points (such as end of consolidation) identify ALL subgroups which deserve investigation of more effective treatments. Recently, new MRD markers have been identified which can be incorporated to improve the applicability and sensitivity of FCM-based MRD monitoring. The predictivity of MRD as a measurement of drug response *in vivo* may open new perspectives for its use in clinical decisions, and possibly as a surrogate for efficacy in the evaluation of novel therapeutic approaches.

FLOW CYTOMETRIC DETECTION OF ORAL PATHOGEN BACTERIA

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Flow cytometry, in combination with fluorescent probe techniques, has been successfully applied for the rapid and specific detection and enumeration of bacteria. Its use has thus become an exceptionally powerful tool for investigating characteristic features of individual cells in heterogeneous populations.
In particular, in this study, a flow cytometric approach was used in order to detect four bacterial species (*S. mutans* ATCC 25175, *S. oralis* ATCC 9811, *F. nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277) responsible of human oral diseases. To this purpose, polyclonal antibodies against these oral species were produced and tested for sensitivity and specificity.
Results revealed high sensitivity for each antibodies. The specificity of each antibodies was tested towards other oral bacterial species. Moreover, to reduce the eventual cross-reactivity, each polyclonal antibody was co-adsorbed with different mixtures of oral microorganisms.
Furthermore, we tested some antimicrobial agents such as carvacrol, a monoterpenic phenol with several medicinal properties, and chlorhexidine, which has different biological properties, in order to investigate their antimicrobial activity against *S. mutans* ATCC 25175, *S. oralis* ATCC 9811, *F. nucleatum* ATCC 25586, *P. gingivalis* ATCC 33277. Results evidenced that the exposure to carvacrol and chlorhexidine for different times caused significant effects on membrane integrity of all oral pathogens, suggesting that this essential oil may be useful for the prevention and treatment of periodontal diseases.
In conclusion, the application of flow cytometry in combination with specific polyclonal antibodies and some fluorochromes could be a helpful method for the analyses of bacteria also in heterogeneous samples.

FLOW MOLECULAR CYTOGENETICS: FLUORESCENCE *IN SITU* HYBRIDIZATION IN SUSPENSION (FISHIS) OFFERS NEW CHANCES TO FLOW SORTING OF CHROMOSOMES AND NUCLEI

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Flow cytometry and cell sorting are techniques of growing relevance for their analytical accuracy and high quantitative throughput which couple well with the powerful next generation sequencing (NGS) technology which has been opening new avenues in genomics. But large and/or polyploid genomes are still a serious task to decipher since the huge amounts of repetitive sequences, which they are made of largely, are still difficult to assemble in a proper and truthful way. Plants, and most of the industrial crops such as wheat, cotton, banana, potato, coffee, have polyploid and/or large genomes which make the discovery of the their DNA sequencing a daunting task. Therefore, the "chromosome approach", or isolating chromosomes or chromosome arms via flow sorting according their different sizes, offers a clue to resolve such a complexity by focusing sequencing to a discrete and self-consistent part of the whole genome. But the standard chromosome complement is not so polymorphic to permit

a direct flow sorting of single type chromosomes and this approach is rather limited to very few plants where cytogenetic mutants are available enabling enough chromosome discrimination by size. We present a reliable, fast and cost effective method for Fluorescence *In Situ* Hybridization In Suspension (FISHIS) of plant chromosomes that overcomes this limitation allowing chromosome sorting based on total DNA content (size) but also on a FISH specific chromosome labeling pattern. FISHIS relies on alkaline DNA denaturation and on chromosome hybridization with readily available synthetic, fluorescently labeled, repetitive sequences as probe. We show that our method specifically labels and lets flow sorting of several chromosomes from standard plant karyotypes. Given the ubiquitous occurrence of used repetitive sequences, the abundance of their variants and their overall chromosome-specific distribution, FISHIS experiments potentially open the access to virtually any complex eukaryotic genomes and chromosomes.

TUMOR METASTASIS: EMT, STEMNESS AND MIGRATION

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The value of cell sorting strategies for cancer research stems from the tumor heterogeneity and inability of other research approaches to resolve phenotypic and genomic differences in cancers. Solid tumors are composed of different cell subpopulations which have different functions in morphological, transcriptional, epigenetic and genetic traits. Flow cytometry activated sorting (FACS) allows for isolation and purification of cancer cells for subsequent analysis (such as PCR, RNA or protein microarrays; Western blot, Southern blot, functional clonal assays), and highly selective enrichment for stem cell autologous transplantation. Moreover, FACS made possible isolation of enriched cancer stem cells (CSCs), sorting of small populations (and rare cells with frequency of 0.03%) for xenograft transplantation, and sorting single cancer cells for single-cell studies.
Metastasis is the leading cause of death by cancer and remains poorly understood due to its complexity. It involves many steps: local invasion of cancer cells into the surrounding tissue, transport through the microvasculature of the lymph and blood systems, translocation, mainly through the bloodstream, to microvessels of distant tissues, exit from the bloodstream, survival and adaptation in the distant microenvironment, and, finally, formation of a secondary tumour. Recent researches have demonstrated that epithelial-to-mesenchymal transition (EMT) plays a key role in the early process of metastasis of cancer cells. Transforming growth factor-β1 (TGF-β1) is the major inductor of EMT. In this context, aim of this study is to use FACS for isolation of CSCs as cells positive for CD133 marker and Side Population (SP) and non-CSCs as cells negative for CD133 marker and SP, to treat them with TGF-β1 and, then, to investigate their role in migration starting from a A549 lung cancer cell line.
To isolate CSCs and non-CSCs starting from the A549 cell line, we sorted both for CD133 expression and SP parameters. We found that the mean expression levels of CD133 were about 4%. The presence of SP cells in the A549 cell line was examined by staining cells with Hoechst 33342 to generate a Hoechst blue–red profile. On the basis of the variance in Hoechst 33342 labeling, we gated the strongest dye efflux cells as SP+ cells, which were in the lower left quadrant of the FACS profile. As a control, the ATP-binding cassette (ABC) transporter inhibitor verapamil was added to reduce the capacity to exclude Hoechst 33342. SP cells were detected in A549 cells, with a mean percentage of about 15% of the total cell population, and the SP cell fraction was abolished in the presence of verapamil. The purity of sorted populations was routinely 95% and sorted cells both for CD133 and for SP were used for TGF-β1 treatment assay, RT-PCR, immunofluorescence and in vitro migration assays. We demonstrate that TGF-β1 induced EMT in both CSC and non-CSC A549 sublines, up-regulating the expression of mesenchymal markers such as vimentin and Slug, and down-regulating levels of epithelial markers such as e-cadherin and cytokeratins. CSC and non-CSC A549 sublines undergoing EMT showed a strong migration and strong levels of MMP9 except for the $CD133^-$ cell fraction. OCT4 levels are strongly up-regulated in all cell fractions except $CD133^-$ cells. On the contrary, wound size reveals that TGF-β1 enhances motility in wild-type A549 as well as $CD133^+$ and SP^+ cells. For $CD133^-$ and SP^- cells, TGF-β1exposure does not change the motility. Finally, assessment of growth kinetics reveals major colony-forming efficiency in $CD133^+$ A549 cells. In particular, SP^+ and SP^- A549 cells show more efficiency to form colonies than untreated corresponding cells, while for $CD133^-$ cells no change in colony number was observable after TGF-β1 exposure. We conclude that it is possible to highlight different cell subpopulations with different grades of stemness: cells expressing CD133 could be a true migrating subpopulation in lung adenocarcinoma while SP^+ cells could be considered the stationary CSC subpopulation. Then, we hypothesize also that SP^- cell fraction could represent a potential stem subpopulation used by tumours as a reservoir of stem cells in certain conditions.

INNATE IMMUNITY AND TUMOR PROGRESSION: A FLOW CYTOMETRIC POINT OF VIEW!

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The last decade witnessed an ever-growing awareness of the promoting role of chronic inflammation in cancer initiation and progression. Cancer-related inflammation is now recognized as a hallmark of tumors. Tumor-associated macrophages (TAM) and myeloid cells (monocytes and Myeloid-Derived Suppressor Cells) are present in large numbers in tumor tissues and are key promoters of both angiogenesis and suppression of adaptive immune responses. Moreover clinical and experimental evidence has shown that high density of these leukocyte subsets is associated with poor patient prognosis and resistance to therapies.
Interestingly several studies have shown that tumor progression can lead to extramedullary haematopoiesis that causes changes in leukocyte compositions in several organs like bone marrow, blood and spleen, while at the tumor site the increase of myelo-monocytic cells parallels with necrosis due to the growth of the tumor mass. This complex framework requires the use of different antibodies for a correct flow cytometric analysis, in the presence of dyes able to exclude dead cells. The immunophenotypic analysis of myeloid cell subsets through flow cytometry both in humans and murine models by a multiparametric approach involves the use of the right combination of fluorophores in order to minimize the compensation and the aspecific signal derived from the high autofluorescence of these cells.

Indeed setting up a reproducible and efficient method for myelo-monocytic cell detection through flow cytometry should contribute to understand the complex cross-talk between the tumor and the immune system.
In addition, the targeting of myeloid cells in tumors is considered a promising therapeutic strategy: the depletion of TAM or their "re-education" as anti-tumour effectors is currently under clinical investigation and hopefully will contribute to the efficacy of conventional anti-cancer treatments.

Cell Cycle and Apoptosis

CONVENTIONAL AND MULTISPECTRAL IMAGING FLOW CYTOMETRY: INTEGRATED ANALYSIS OF APOPTOSIS

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Apoptosis is a complex multi-step process. The assay most commonly used to discriminate between viable and dead cells is based on the staining with Annexin-V and a cell-impermeant nuclear dye. This analysis considers only one of the hallmarks of apoptosis: the exposure of phosphatidylserine on cell surface. However, there is the increasing need to evaluate also other significant events such as caspase activation, DNA fragmentation and the balance between pro- and anti-apoptotic proteins. Multispectral Imaging Flow Cytometry (MIFC) combines the capabilities of microscopy and flow cytometry integrating the phenotypic data with information about cell and nuclear morphology, protein localization and protein-protein interaction. Considering these peculiarities, we recently combined Conventional Flow Cytometry (CFC) and MIFC to better characterize the molecular mechanisms underlying the apoptotic effects of retinoic acid (RA) plus interferon-α (IFN-α) in mantle cell lymphoma. We demonstrated that the treatment triggers both intrinsic and extrinsic apoptotic pathways and caused Bak and Bax conformational chenges. We also showed that RA/IFN-α induced the expression of Noxa and, taking advantage of MIFC, we demonstrated that the upregulation of this protein was associated with the nuclear translocation of the transcription factor FOXO3a. Finally, given the up-regulated Noxa co-immunoprecipitated with Mcl-1 and Bfl-1, sequestering them from complexes with the apoptotic effector Bid, we analyzed their interaction *in vivo*. In details, we detected Mcl-1/Bid and Bfl-1/Bid co-localization in viable cells by MIFC, revealing that this mechanism is important to prevent Bid activation.
In conclusion, our findings indicate that the integration between CFC and MIFC may allow a more precise identification of critical mediators of drug-induced apoptosis, leading to a more in-depth understanding of their regulation and interaction with specific partners of the complex apoptotic pathways.

MULTIPLE EFFECTS OF A SODIUM/HYDROGEN ANTIPORTER INHIBITOR

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Amiloride derivatives are new promising chemotherapeutic agents. A representative member belonging to this family is the sodium-hydrogen antiporter inhibitor HMA

(5-(N,N-hexamethylene amiloride), which induces cellular intracytosolic acidification and activates apoptosis. We characterized the response to HMA of the SW613-B3 colon carcinoma cell line that shows an intrinsic resistance to a panel of drugs, showing that HMA is able to induce multiple effects. First, we detected that HMA impairs cell survival in a dose dependent manner and in an irreversible way; then, we analyzed cell morphology and we observed that cell number appear to be reduced in a dose-dependent manner and surviving cells were condensed; also the cytoskeletal pattern was dramatically altered. Thus, we pointed out the possible effects of this drug on DNA; we demonstrated that HMA is able to induce alteration in cell cycle distribution, leading to a G_2/M block; furthermore, DNA damage was evidenced by the comet assay, which revealed the presence of a considerable amount of DNA damage, both as SSBs (single strand breaks) and oxidized pyrimidine or purine. We finally identified that in response to HMA, these cells activate programmed cell deaths, which are independent of caspases activation (like parthanatos and LEI/L-DNase II pathway). In addition, also autophagy occurs in our experimental system, thus supporting the idea that drug response is characterized by the firing of several death pathways, which often are co-regulated.

PROLIFERATIVE EFFECT OF LOW-LEVEL LASER IRRADIATION ON BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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Low-level laser irradiation (LLLI) was found to increase proliferation potential of various types of cells *in vitro*. However, the mechanisms associated with this phenomenon remain poorly understood. Human bone marrow-derived mesenchymal stem cells (hBMSCs) have shown to be an appealing source for cell therapy and tissue engineering. In this study we examined the *in vitro* effect of a single or a multiple doses of LLLI on proliferation of MSCs isolated from adult human bone marrow of different donors. The cells were irradiated with a red wavelength diode laser (659 nm) and exposed to different laser doses (0.5, 2, 5 J/cm²) for 1 day and 3 consecutive days. Proliferation was evaluated using a quantitative assay. Both single and multiple doses of LLLI enhanced the proliferation of hBMSC isolated from all donors. A significantly cell growth increase was detected in groups irradiated for three consecutive days compared to a single dose or control group. Colony Forming Unit-Fibroblast Examination and Immunofluorescence Analysis of stem cells markers showed that laser treatment did not change their stemness characteristics. These preliminary results showed that LLLI affected positively hBMSCs proliferation *in vitro* and it may have an important impact for the use of mesenchymal stem cells in regenerative medicine: in fact, LLLI may be use as a "photoceutical" bioreactor for *in vitro* stem cells preconditioning prior to transplantation. In this context, our final research aim is understand when and how physical stimuli can be useful for promoting regeneration also for stem cells seeded and cultured on biomaterials.

EFFECTS ON NEUROBLASTOMA CELL CULTURES OF CISPLATIN AND PtAcacDMS (a new platinum compound)

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Cisplatin (cisPt) is one of the most active anticancer drug used in chemotherapy against several types of solid tumours. It binds DNA, forming adducts and blocking fundamental cellular processes, such as transcription and translation. It has also recently been shown that it acts at cytoplasmic level, by damaging organelles and inducing cell death. Despite the great efficacy, cisPt has several side effects, such as nephrotoxicity and ototoxicyty and induces drug resistance, which greatly compromise its healing potential. For this reason it became necessary to find some new platinum-based drugs, which could be able to overcome these limits. A new platinum compound [Pt(*O,O'*-acac)(gamma-acac)(DMS)] (PtAcacDMS) was synthesized; it reacts preferentially with protein thiol or thioether groups, suggesting that its targets are cytoplasmic instead of nuclear. The aim of our research was to compare the ability of cisPt and PtAcacDMS to drive cell death in neuroblastoma cell cultures and to investigate the intracellular mechanisms of *in vitro* cytotoxicity induced by these platinum compounds. Our data show that both cisPt and PtAcacDMS cause apoptosis by different pathways: extrinsic (soluble molecules interact with specific receptors on the cellular membrane triggering death signal transduction) and intrinsic (triggered by intracellular stimuli or DNA damage), both inducing the activation of some specific cytosolic proteases, the so-called caspases. These proteins have several targets inside the cell, such as proteins and factors involved in cell structure maintenance or enzymes controlling DNA metabolism. In previous data we showed the higher toxic potential of PtAcacDMS on cells respect cisPt: as a matter of fact the apoptotic index is higher after PtAcacDMS administration at a concentration four times lower than cisPt (10μM PtAcacDMS vs 40mM cisPt), suggesting that this new compound exerts a more relevant cytostatic action. We also investigated the apoptosis/autophagy ratio by treating cells for 48h with cisPt or PtAcacDMS and 7 days of recovery (drug-free medium), followed by a "replantation" in new flasks. Cells treated with cisPt exhibited autophagic characteristics, while cells processed through the same treatments, but with PtAcacDMS, were apoptotic. In conclusion, cisPt induces autophagy, a possible form of drug resistance, while PtAcacDMS seems to lead tumour cells to death, showing antineoplastic effectiveness *in vitro.*

CELL CYCLE AND SURVIVAL PERTURBATIONS INDUCED BY NEUTRON AND GAMMA IRRADIATIONS: A COMPARATIVE METHODOLOGICAL STUDY

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Boron Neutron Capture Therapy (BNCT) is a non conventional binary radiation therapy for cancer treatment based on the selective accumulation of ^{10}B carriers in tumor followed by neutron irradiation. The highly ionizing particles, a-particles and ^{7}Li ions, generated by the neutron capture in ^{10}B, dissipate their energy in a short range, comparable to the cell diameter. The radiation lethality is therefore confined to the neoplastic boron enriched cells while the surrounding healthy tissue is spared. This peculiarity makes BNCT a selective and effective therapeutic approach expecially in case of disseminated tumors.
The present study was carried on in the frame of the preclinical in vitro validations aimed to verify the BNCT efficacy and applicability to liver or lung coloncarcinoma metastases and to limb osteosarcoma. A comparative cell survival and cell cycle study was performed between adherent and suspended coloncarcinoma and osteosarcoma cell lines, in order to evaluate the alterations induced by thermal neutron and gamma irradiations. In case of neutron irradiation analyses were performed on boron loaded and unloaded coloncarcinoma and osteosarcoma cell lines.
Cell survival data, assessed by cloning assay, evidenced that: i) a cell line-dependant radiosensitization can be noticed in case of adherent cells exposed to both radiation types, being the coloncarcinoma cell line the less damaged one; ii) adherent cells are much more sensitive to radiations than suspended cell.
Cell cycle cytofluorimetric DNA analyses showed an evident dose dependant block in G2 phase for cells irradiated in the suspended condition. Conversely, no evidence of cell cycle delay was observed in case of adherent cells.
The different behaviour between adherent and suspended cells when exposed to radiations must be considered in the evaluation and comparative studies of BNCT induced damages.

FUNCTIONAL CHARACTERIZATION OF A VEGF MIMETIC PEPTIDE BY MEANS OF FLOW CYTOMETRY

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Angiogenesis consist of a series of interactive events that include endothelial cell differentiation, proliferation and migration, which lead to form new vessels. VEGFRs are tyrosine kinases receptors implicated in process of angiogenesis, focus of different study aimed at developing suitable molecules for regulating angiogenesis.
In previous studies, we designed, synthesized and characterized a peptide involved in receptor recognition, QK peptide. This molecule reproduces the α-helix 17–25 of the VEGF binding interface. In vitro biological assays suggested that QK shares numerous biological properties of VEGF.
Here we report the biological characterization of a QK analogue peptide, named IQ. We demonstrated the specific binding of peptide to VEGFR-2 by flow cytometry. Moreover, we showed that it is able to induce endothelial cell proliferation in a dose dependent manner and to rescue cells from apoptosis. These findings indicate that IQ plays a role on endothelial cell signalling, showing a VEGF-like behaviour and provides the basis for a rational design of a novel peptide-based drug for medical applications in therapeutic angiogenesis.

A FLOW CYTOMETRIC ANALYSIS OF MITOCHONDRIAL CELL DEATH IN HGFs/*S. mitis* CO-CULTURE MODEL

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Resin monomers like TEGDMA (triethylene glycol dimethacrylate) enhance the formation of reactive oxygen species (ROS) in human gingival fibroblasts (HGFs) and this event seems to be associated with apoptosis. The acute depletion of mitochondrial GSH exerted by TEGDMA induces ROS production, collapse of the mitochondrial membrane potential (MMP) and cell death. Our work aims to understand the mechanisms that underlie mitochondrial apoptosis in HGFs co-cultured with *Streptococcus mitis,* a microorganism usually present in the oral cavity, in the presence of saliva and treated with 1 mM TEGDMA. Different flow cytometric analyses were carried out: reactive oxygen species detection by CM-H_2DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester), TMRE (tetramethylrhodamine, ethyl ester) assay to label active mitochondria, Annexin V/PI detection of apoptotic and necrotic cells and imaging flow cytometry analysis of BAX intracellular expression. Our results suggest that, in HGFs treated with TEGDMA, the presence of *S. mitis* and/or saliva reduces ROS production, BAX expression and apoptotic cell percentage while, on the other hand, increases MMP potential. In the presence of N-acetylcysteine 10 mM, a well known non-enzymatic antioxidant, the apoptotic population is reduced, mainly in the presence of saliva and bacteria. These results, shedding more light on the biological and molecular events that occur upon TEGDMA treatment *in vitro* in a co-culture model that mimics the environment of the oral cavity, award a key role to oral bacteria and saliva in preventing oxidative stress-induced mitochondrial cell death in HGFs.

RAPAMYCIN (RAPA) PREVENTS LPS-INDUCED ENDOTHELIAL CELL DYSFUNCTION

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Introduction and aims: Endothelial cells (ECs) activation has a pivotal role in sepsis-induced AKI. Sepsis may induce phenotypic alteration of ECs by several mechanisms. The aim of our study was to evaluate the effects of LPS on ECs and the role of RAPA in the modulation of endothelial dysfunction.
Methods**:** After 24h or 48 of LPS activation, ECs were

analyzed by MTT cell viability assay and flow cytometry analysis.
Results: Through MTT assay, we observed a significantly proliferation of ECs after LPS activation LPS 4□g/ml (p= 0.04). When we addedd RAPA (5 nM) we found that the cell growth was restored to the basal condition by inhibiting LPS-induced activation (p = 0.05). Flow cytometry analysis (AnnV / IP) showed that only a small percentage of ECs underwent to apoptosis after LPS activation (8.9% ±1.6 vs basal 5,9% ± 1.2). Moreover, the addition of RAPA did not induce apoptosis in LPS activated ECs. Interestingly, LPS-activated ECs showed a reduction in endothelial markers such as CD31 (66.46% ± 4.69 vs basal: 90.6% ± 4.12, p=0.003) and VE-cadherin (23.8% ± 5.24 vs basal: 54.4% ± 4.26, p=0.003) and an increased expression of fibroblast markers N-cadherin (66.8% ± 4.84 vs basal: 8.46% ± 1.4, p=0.004), Vimentin (60.27% ± 7.44 vs basal: 40.69% ± 2.24, p=0.03) and FSP-1 (53,54% ± 6.81 vs basal: 26.47% ± 5.05, p=0.04). Interestingly, the addition of RAPA abrogated the LPS-induced endothelial dysfunction by restoring the expression of CD31 and VE-cadherin, and inhibiting the expression of de-differentiation markers such as N-cadherin, Vimentin and FSP-1.
Conclusions: These data suggest that LPS acts directly on ECs by activating cellular proliferation and promoting endothelial dysfunction and de-differentiation. The inhibition of ECs activation via RAPA may represent a possible therapeutic strategy to prevent endothelial dysfunction during sepsis-induced AKI.

LEPTIN INDUCES THE GENERATION OF MICROPARTICLES BY HUMAN MONONUCLEAR CELLS

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Purpose: Microparticles (MP) are phospholipid vesicles shed by cells upon activation or during apoptosis. MP are involved in numerous physiological processes, including coagulation and inflammation. Leptin, synthesized by adipose tissue, has been implicated in the regulation of inflammation and the pathogenesis of thrombosis.
Aim: Aim of our study is to test the hypothesis that one of the mechanisms of leptin is linked to the induction of procoagulant MP by human mononuclear cells (HMCs) and to investigate the intracellular mechanisms leading to MP release upon incubation with leptin.
Methods: HMCs are isolated from the peripheral blood of healthy donors. Cells are incubated with leptin (10 µg/mL: 4 hours). Leptin-stimulated cells are also incubated with a phospholipase C inhibitor, U73122 or with three different inhibitors of MAP kinases. MP generation is assessed as phosphatidylserine (PS) concentration by a prothrombinase assay, after capturing the MP onto annexin V-coated wells and by cytofluorimentric analysis. HMCs derived MP were discriminated first by size, as events conforming to a light scatter distribution within the 0.5-0.9 μm bead range in a SSC vs. FSC window and further identified as CD14 and annexin V positive events after incubation with fluorescein isothyocianate (FITC)-annexin V and allophycocyanin (APC)-anti CD14 Ab, in a APC vs. FITC window.
Results: Leptin significantly stimulates MP generation by HMCs (0.38±0.12 vs 0.21±0.11 nM PS; mean±SEM). These results are confirmed by cytofluorimetric analysis (262±51.39 vs 38.67±10.09 events CD14+/annexinV+). U73122, 1µM, significantly inhibits leptin-induced MP release (0.32±0.15 vs 0.46±0.22 nM PS). MAPK inhibitors have no effect.
Conclusions: Leptin induces the release of MP with a procoagulant potential by HMCs through a mechanism that involves phospholipase C activation. These data are consistent with a role of leptin-induced procoagulant MP shed by MNCs in vascular diseases linked to obesity.

RUBINSTEIN-TAYBI SYNDROME LYMPHOBLASTOID CELLS SHOW INCREASED SENSITIVITY TO OXIDATIVE DNA DAMAGE

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The Rubinstein–Taybi syndrome (RSTS) is a genetic disorder associated with defects in postnatal growth, microcephaly, intellectual disability and increased risk of tumors. The exact molecular etiology of RSTS is not clearly understood; however it is widely accepted that RSTS is associated with mutations and chromosomal rearrangements (microdeletions and translocations) with breakpoints at 16p13.3, where the gene encoding the cAMP Response Element-Binding Protein (CREBBP or CBP) is located. About 60% of RSTS individuals carry a heterozygous mutation/deletion of CREBBP, while a small percentage (~3%) of RSTS are caused by mutations in the EP300 gene (located at 22q13.2) encoding for p300. CREBBP and p300 are highly homologous proteins with lysine acetyl transferase (KAT) activity, and play a key role in transcription regulation. However, they have also distinct cellular functions and cannot always replace one another. Both proteins are involved in other aspects of DNA metabolism, including cell cycle checkpoints (by acetylating p53), and DNA repair (by acetylating factors involved in nucleotide (NER) and base excision repair (BER) mechanisms, such as XPG, FEN-1, DNA polymerase β (pol β), some DNA glycosylases (e.g. TDG, NEIL2, OGG1), as well as PARP-1, and WRN and RECQL4 helicases. In agreement with these findings, we have recently shown that depletion of both CBP and p300 by RNA interference, significantly impairs NER. Thus, deficiency in CBP and/or p300 may result in genome instability and cancerogenesis, thereby explaining, at least in part, the higher incidence of malignancies observed in RSTS patients. In order to study the involvement of CBP and p300 in response to DNA damage, and in particular in DNA repair, we have analyzed lymphoblastoid cell lines derived from RSTS patients carrying monoallelic deletion, or mutations of CREBBP gene. Flow cytometry and BrdU labeling has been used to assess proliferative characteristics of these cell lines. Western blotting analysis has shown, as expected, a reduced expression of CBP, and in one case also of p300 protein in RSTS lymphoblasts, as compared to the lymphoblastoid cell lines from normal donors. No significant differences were observed in the protein expression of PCNA, and other related DNA repair proteins, such as XP proteins (A-G), PARP-1, XRCC1 and DNA pol β. Despite a similar proliferation rate, RSTS cells showed signs of histone H2AX phosphorylation, suggesting the presence

of endogenous DNA damage. In addition, all RSTS cell lines tested were more sensitive to treatment with the oxidative agent $KBrO_3$. Experiments have been performed to assess the recruitment of XRCC1 and DNA pol β to DNA damage sites, and the efficiency of DNA repair is being analysed by the Comet test. Preliminary results suggest a different and delayed kinetics in the repair of oxidative lesions in RSTS cells; however, further investigations are required in order to fully characterize the behaviour of these cell lines.

Environmental Sciences and Toxicology

AN INTEGRATED APPROACH TO UNDERSTAND THE MICROBIAL COMMUNITY STRUCTURE AND FUNCTIONING IN COASTAL AREAS

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In November 2010, 32 stations were sampled to estimate heterotrophic bacteria and picophytoplankton abundance by flow cytometry, bacterial community composition and metabolic activity by CARD-FISH and MAR-CARD-FISH. Prochlorococcus was the most abundant autotrophic picoplankter (1.88 ± SD $1.10x10^4$ cell ml^{-1}), followed by Synechococcus (1.50 ± SD $1.07x10^4$ cell ml^{-1}) and picoeukaryotes (7.59± SD 6.20 $x10^2$cell ml^{-1}). These showed the highest concentrations in the Sele river estuary at surface ($1.98x10^4$ cell ml^{-1},$4.89x10^4$ cell ml^{-1}, $3.31x10^3$ cell ml^{-1}, respectively). Heterotrophic bacteria were 6.01 ± SD 3.88 $x10^5$cell ml^{-1}. Their concentrations varied from $1.48x10^5$ and $2.17x10^6$ cell ml^{-1} following the coastal offshore gradient with higher concentrations near the rivers confirming their role as remineralizers of organic matter and nutrient recyclers. Bacterial community composition was analysed by CARD-FISH. 75% ± of total cell counts were detected with the EUB 338 probe. The overall most abundant group was Alphaproteobacteria 56% ± 6.7. The second group in terms of abundance was Cytophaga-Flavobacterium-Bacteroides 30% ± 5.4. Gammaproteobacteria were on average 22% ± 5.0. Along the Sarno and the Volturno river Cytophaga and Gammaproteobacteria showed an increase from the offshore stations (22% ± 2.1 and 19% ±1.8 CFB and Gamma, respectively) to the coastal ones (30% ±1 and 24% ± 2 CFB and Gamma respectively). On the contrary Alphaproteobacteria showed an opposite trend with higher concentration offshore (55% ± 2.0) and lower near the coast (44% ± 3.2). Among the Alphaproteobacteria, SAR11 was the most abundant clade (up to 30% of total Eubacteria counts). The least abundant clade at all stations was Roseobacter (9% ± 3.4). These data are to be complemented with information from two metagenomic sequences with the aim of providing integrated tools for the assessment of biological properties of coastal areas.

EFFECT OF ALGAL POLYUNSATURATED ALDHEYDES ON A NATURAL MARINE BACTERIAL COMMUNITY FROM THE GULF OF NAPLES

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Polyunsaturated aldehydes (PUA) are algal secondary metabolites toxic for different marine organisms. The effect of different PUA on community composition and activity was tested on natural heterotrophic bacterial communities in July 2009. Triplicate surface samples were incubated with heptadienal (HEPTA), octadienal (OCTA), decadienal (DECA) and a mix of the last two (MIX) and assayed after 6 and 24 h. Initial bacterial abundances, estimated by flow cytometry, were 8.98 x 10^5 ± SD 2 x 10^5 cell ml^{-1} and did not vary significantly during

the experiment, probably due to a compensatory effect. Community composition and activity was estimated by CARD-FISH and MAR-CARD-FISH. After 6 h incubation, Gammaproteobacteria (Gamma), CFB and ROS clades were not affected by PUAs and even were stimulated differentially by some of the PUAs tested (up to 59% for Gamma with MIX). Alphaproteobacteria (Alpha) were the most affected, with a dramatic decrease of 83%, while SAR11 was not affected. The stimulatory effect was confirmed after 24 h for Gamma and CFB, but not for ROS. Alpha recovered and even increased their contribution despite the fact that the control had lowered in percentage. At the end of the experiment, the community composition had changed and mostly profited Gamma which increased their relative contribution from 19% to 38% relative to the total Eubacteria which increased, in turn, from 85% to 100%. The same was reflected by the MAR-CARD-FISH data, with Gamma, CFB and ROS showing a higher activity when compared to the t0 and the relative control after 6 h exposure, especially with the MIX, while Alpha were initially affected but recovered after 24 h. The metabolic activity of SAR 11 was not affected by any treatment. These data confirm previous results where Gamma was also the least affected by PUA and the MIX had a stronger positive or negative effect than the single PUA, suggesting a synergistic effect. The role of PUA in shaping the bacterial community is confirmed, with differences depending from factors yet to be elucidated.

DISTRIBUTION AND COMPOSITION OF NATURAL BACTERIAL COMMUNITY AT A LONG TERM ECOLOGICAL RESEARCH STATION (LTER) IN THE GULF OF NAPLES

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Bacteria are important components of pelagic food web both in terms of biomass and activity. Their abundance, distribution and taxonomic composition were studied during 2011 at the Long Term Ecological Research Station MareChiara, in the Gulf of Naples. Bacterial abundance was estimated by flow cytometry and community composition by CARD-FISH. Heterotrophic bacteria were homogeneously distributed along the water column during winter as a consequence of seasonal mixing. The highest concentrations were recorded in spring (2.72 x 10^6 cell ml^{-1} in May), as well as in summer and autumn (2.70 x 10^6 cell ml^{-1} and 2.17 x 10^6 cell ml^{-1} in August and November, respectively). These peaks followed chlorophyll a and phytoplankton abundance distribution, likely to indicate that the organic matter released by phytoplankton was being utilized for bacterial growth.
71% (±4%) of heterotrophic bacteria were detected using the Eubacteria probe by CARD-FISH. Among these, the most abundant group was Alphaproteobacteria (50%±5) with a peak in March (59% ±13%). This group is known to remineralize nutrients used by phytoplankton, which well correlates with the abundance and chl a data. Within the Alphaproteobacteria, SAR11 was the most abundant group along the year, showing an opposite seasonal trend to Roseobacter, which was generally found in lower proportions. During the year Bacteroidetes and Gammaproteobacteria were on average 22% (±5%) and 21% (±6%), respectively. Bacteroidetes were the most abundant group in April, maybe due to the lower water temperature. This is possibly due to the fact that this bacterial group is more efficient in using different organic compounds at low temperatures in coastal waters. On the contrary, Gammaproteobacteria increased up to 50 % during February due to external input. Altogether, these data indicate that, at the LTER MareChiara, bacterioplankton abundance and composition depend on different environmental factors, including temperature, salinity, nutrients and chlorophyll a.

DETECTING SPERM DNA FRAGMENTATION TO DISCRIMINATE BETWEEN FERTILE AND INFERTILE MEN

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Sperm DNA Fragmentation (sDF) is an anomaly of sperm genome consisting in single and double stranded DNA breaks. The impact of sDF on reproductive outcomes remains elusive due to the conflicting results of clinical studies. The ability of tests detecting sDF to predict the outcomes of reproduction is affected by many variables, including the sperm population where the damage is revealed. Using TUNEL/PI, coupling the detection of sDF to the nuclear staining with propidium iodide, PI, our group unveiled two flow cytometric sperm populations that differ for PI staining (termed PI brighter and PI dimmer populations), for the amount of sDF and for cell viability. Indeed, PI dimmer sperm are all DNA fragmented and not viable. Conversely, PI brighter sperm are both fragmented and not fragmented and both viable and not viable. Based on this finding we reasoned that PI dimmer sperm have no chance to participate in fertilization, that the fraction of sDF really impacting on reproduction is that of PI brighter sperm and, within it, that of viable gametes. To verify this hypothesis, we set up a method able to detect sDF in viable spermatozoa by using a LIVE/DEAD fixable stain that labels dead cells permanently, thus remaining even after processing samples by TUNEL for sDF detection. Then we compared the levels of sDF as measured in total, PI brighter and live spermatozoa in 23 fertile and 22 infertile men. As expected, we found that sDF resulted increased ($p<0.05$) in infertile respect to fertile subjects, both in total (44.6±18.8 vs 35.2±13.6%) and PI brighter (33.2±15.8 vs 24.3±10.8%) and live sperm (25.1±19.3 vs 13.6±6.2%). However, the percentage increase in infertile vs fertile subjects was much greater for viable sperm (84.4%) respect to PI brighter sperm (36.4%) and total population (26.5%). In conclusion, the ability of sDF to discriminate between fertile and infertile men, ameliorates considering PI brighter and above all viable sperm, respect to total sperm population.

NANOFIBRILLAR CELLULOSE INDUCES CYTOTOXIC EFFECTS IN HUMAN LEUKOCYTES

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Nanofibrillar-cellulose (NFC) based materials have attracted a growing interest in different fields of applications such as packaging, adhesives, fibers, opto-electronic and medical devices, and automotives components for their innovative properties. For some alimentary and medical use it is mandatory to verify its biocompatibility, namely its biological acceptability. The basic nanosafety tests verify if a material, its extracts or suspensions induce cell death, malformation, degeneration and lysis. To check possible cytotoxic effects, flow cytometric techniques and Electron Scanning Microscopic (ESEM) observations were applied to investigate toxic effects on human leukocytes (lymphocytes and granulocytes) after NFC exposure.

Different NFC samples called CTRL, KJ, TEMPO were administrated to the cells at different times (T=6h, 24h, 48, 72h) and analysed by flow cytometry in order to evaluate cell death (propidium iodide uptake), membrane potential (TMRE), mitochondrial cardiolipine content (NAO) and acidic compartment (AO).

Results show a rise in apoptotic and necrotic cell number especially on granulocytes, for CTRL at 6h and 24h; while TEMPO treatment induces a progressively increasing cell death during all time course.

ESEM observations confirm the cytometric data, especially for the reduction of the cell number. focusing flow cytometric approach as an extremely appropriate and useful tool to detect cytotoxic effects.

Our results indicate that NFC suspensions (mainly CTRL and TEMPO samples) are proficient cytotoxic inducers, in both number of cells involved and in degree of damage induction, suggesting that these nanostructured materials are probably not suitable to be used for food packaging and medical devices.

CYTO-GENOTOXICITY AND INFLAMMATORY RESPONSE IN HUMAN ALVEOLAR (A549) CELLS EXPOSED TO TITANIUM DIOXIDE NANOPARTICLES

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The increasing use of titanium dioxide nanoparticles (TiO_2NPs) in consumer products (cosmetics and food additives) and industrial and medical applications highlights the importance of understanding its potential toxic effects. Since the respiratory tract represents an important exposure route for workers employed in the TiO_2NPs production, we investigated the cyto-genotoxic and inflammatory effects induced by commercial TiO_2NPs in human alveolar cells (A549) exposed to 1, 5, 10, 20 and 40 μg/mL. Cytotoxic effects were studied by viability analysis with WST1 test after 24h exposure and cell membrane damage by LDH release assay after 30 min, 2 and 24h. Direct/oxidative DNA damage was assessed by Fpg-modified comet assay and the inflammatory potential by IL-6, IL-8 and TNFα release by ELISA, both after 2 and 24h exposure. We did not find significant cell viability reduction. Slight membrane damage was detected after 30 min and 2h at 40μg/mL. Slight direct DNA damage was found only after 2h exposure at the highest concentration. A moderate induction of oxidative DNA damage was found at 40 μg/mL after 2h and at 5 μg/mL after 24h exposure. A slight increase of IL-6 release was detected only at 5 μg/mL after 2h exposure. No effects on IL-8 and TNFα releases were observed. The results show a lack of cytotoxic effects in terms of cell viability reduction and a slight early membrane damage. No significant direct DNA damage was also found while a slight oxidative DNA damage and IL-6 release was induced. The secretion of pro-inflammatory cytokine IL-6, involved in early acute inflammatory response, and the slight oxidative DNA damage indicate the promotion of mild oxidative-inflammatory effects for this kind of NP on human alveolar cells. Overall the findings confirm the low cyto-genotoxicity of TiO_2NPs and an early inflammatory response of A549 cells, demonstrating the suitability of this experimental system to study nanomaterial-mediated toxicity.

A FLOW MOLECULAR CYTOGENETIC APPROACH TO MAKE SIMPLER COMPLEX GENOME GENOMICS: FISHIS

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Genome complexity of major crop plants, which results from polyploidy and high abundance of non-genic repetitive sequences, has hampered a fast progress in the exploitation of genomic technologies as analytical and even breeding tools. By enabling genome dissection into single chromosomes or chromosome arms via flow sorting, the 'chromosome approach' can contribute to reduce genome complexity. Up till now, this approach is restricted to species, or special cytogenetic stocks, containing chromosome types that differ in size from the standard complement. The chromosome approach proved to be very effective in dividing the large bread wheat genome in small and significant parts, but it has in itself the limitation of being available in the genetic background of a specific variety (e.g. *T. aestivum* Chinese Spring), not one of major interest. Moreover, such an approach is unpractical for the most of plants and animals, precluding an easy development of genome sequencing and assembling in the case of complex genomes. A new method has been developed which overcomes these limitations, termed FISHIS ''Fluorescence In situ Hybridization In Suspension'', allowing the coupling of the high discriminatory capabilities of FISH labeling and the high-throughput of flow-cytometry analysis (FCM) and flow-sorting to isolate pure chromosome and nuclei fractions, regardless of the availability of cytogenetic stocks. FISHIS is a washless method that relies on readily available fluorescently labeled DNA repetitive sequences (e.g. SSRs) and employs alkaline DNA denaturation to overcome all steps causing chromosome clumping and losses during classic FISH. FCM-FISHIS can discriminate, and flow sort: i) the entire chromosome set of a single genome from the homeologous ones in polyploid pasta and bread wheat; ii) several single-type chromosomes in a number of *Triticeae*, and iii) the entire chromosome complement of the wild grain mosquitograss *(D. Villosum* L.). The FISHIS method potentially extends the analytical and preparative power of flow-sorting to each individual chromosome and nucleus of virtually any eukaryotic species, providing that suitable DNA probes and high quality chromosome suspensions would be both available.

AN INTEGRATED FLOW CYTOMETRY-BASED APPROCH TO SHAPE NEW BEAUTY IN ORCHIDS

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Orchids account for several thousands of species, actually more than 25,000 species, and have a consistent worldwide market which depends largely on the constant availability of new and surprising varieties satisfying the consumers' request for. Therefore, breeding for new varieties and hybrid combinations attractive to the consumers is the main target in orchids. A main constraint to a classical breeding in orchids is their slow growth habit which may delay flowering up to five or more years from the crossing. A possible way to introduce new variability is to double the ploidy level of the wild and commercial varieties; and polyploidy is a well known event which can contribute in ornamental plant breeding. In orchids, polyploids showed larger flowers, extended blooming time and multiple flowering. We have developed in *Dendrobium* a highly regenerative tissue culture system which makes use of PLBs (protocorm like bodies) in liquid culture to obtain a fast growing material to be utilized for polyploidization experiments. Polyploids may be generated through the use of spindle-formation inhibitors without affecting cell-viability, if a proper concentration of blocking agent would be supplemented into the culture substrate. A number of substrates both liquid and solid, were tested combining several spindle inhibitors (COLchicine and APM) with different concentrations and incubation timings. The fresh-weight changes, the regeneration frequency and the ploidy evaluation were all assessed on the PLBs cultures highlighting the good performance of COL as a polyploid agent. Similar efforts were performed on *Phalaenopsis* e *Cattleya*, but with minor results. Ploidy early screening was carried out with flow cytometry (FCM) on nuclei extracted from 10-20 milligrams of plant tissues, both PLBs or young leaftlets, allowing a clear discrimination of the ploidy levels after few weeks of *in vitro* subculturing. Regenerants with different ploidy levels underwent to a molecular characterization with RAPD and SSR showing their different genetic constitution, which was corroborated also by a faster growth, in respect to parentals. A genome-downsizing effect was detected by FCM on several regenerated plantlets since this phenomenon can't be assessed easily by standard cytological observation because of the very tiny size of orchid chromosomes. More than 30 new *Dendrobium* polyploid plantlets were identified and selected after no more than three months of *in vitro* subculturing, allowing a remarkable reduction in working-time and reagent-costs eventually needed to maintain a large experimental population of orchid plants. This work is supported by MIPAAF, Project NOVAORCHID (D.M.11074/7643/09).

FLOW CYTOMETRIC EVALUATION OF INTRACELLULAR UPTAKE AND TOXICITY IN LYMPHOCYTES AND MONOCYTES EXPOSED TO NANO OR MICRO SIZED TiO_2 PARTICLES

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Increasing scientific interest on nanoparticles application is driven by their unique physicochemical properties as reported in several studies carried out in the last few decades. A multiplicity of nanomaterials of various shapes have been engineered for technological improvement in many branches of medicine, electronics as well as generic consumer goods. In parallel, a growing concern about possible health risks associated to nanoparticle exposure has emerged and scientific investigation are in progress. At a cellular level, adverse effects may stem from intracellular uptake of nanoparticles and/or indirect stress response mechanisms.
We analyzed by flow cytometry (FCM), reactive oxygen species (ROS) production and cell viability in immunolabelled subpopulations of human lymphocytes and monocytes exposed to nano or micro sized TiO_2 particles. DNA damage evaluated by Comet assay and 8-oxoG increased levels were observed in unstimulated lymphocytes. Conversely no micronucleus induction was observed in stimulated lymphocytes.
We showed that different levels of toxicity in the two cell types might be directly correlated with different levels of particle uptake measured by FCM scatter signals. In addition, the lower level of uptake in monocytes exposed at 4 °C than in monocytes exposed at room temperature suggested that in these cells uptake occurred by an active endocytosis mechanism.

AN IMPROVED FLOW CYTOMETRIC (FCM) IMMUNODETECTION METHOD TO EVALUATE SPERM DNA GLOBAL METHYLATION LEVEL (DGML)

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DNA methylation is an epigenetic mechanism involved in the regulation of parental imprinting, gene expression, and genomic stability. Aberrant DNA methylation has been associated with aging, diseases and exposure to environmental contaminants potentially toxic for human male reproduction. 5-methyl Cytosine (5-mC) quantitative immunostaining is a cheap method for globally assessing sperm DNA methylation, but the pertinent literature is

scanty and lacks information about quality control and reproducibility of the fluorescence measurements. In the frame of a EU project aimed at studying how contaminants may impact on male reproductive disorders, we optimized and applied a FCM method for 5-mC immunodetection on sperm. The probe consisted of an anti-5-mC mouse monoclonal antibody coupled with AlexaFluor488-conjugated Fab anti-mouse IgG1 fragments. The sperm DGML was evaluated in deep frozen archived samples from 270 healthy fertile men from Greenland, Ukraine, and Poland. Variability of the methodology, evaluated with the help of a reference sample, resulted <15%. Even though this procedure does not stoichiometrically determine the number of 5-mCs in the genome, the green fluorescence intensity mirrors the number of 5-mCs accessible to the antibodies' complex. Sperm DMGL resulted quite independent from conventional semen quality parameters. Partially funded by Project CLEAR "Climate, Environmental Contaminants and Reproductive Health" (www.inuendo.dk/clear), European Commission 7th Framework Programme FP7-ENV-2008-1 Environment (including Climate Change) Grant no. 226217.

THE SIGNIFICANCE OF GENETIC DETERMINANTS OF DETOXIFICATION AND ENVIRONMENTAL RISK-FACTORS IN CHRONIC DISEASES AND CANCER PREVENTION

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Several exogenous and endogenous factors, including inherited differences in metabolic activity, environmental exposures to genotoxic agents, and nutritional habits cause individual predisposition to cancer and chronic diseases.
The cellular mechanisms for detoxification, working principally in two phases, are indispensable to protect cells from damage caused by exposure to a number of environmental toxins and carcinogens. In phase I the substrates are mostly metabolized to highly toxic products, enhancing the significance of the second detoxification step.
Genetic polymorphisms can cause a malfunction in enzyme production resulting in an insufficient amount of enzyme or a reduced activity. They have been detected in a great number of phase I, mainly cytochrome-P450-related enzymes (CYPs), and phase II enzymes as Glutathione-S-transferases (GSTs) and N-acetyltransferases (NATs).
The family of the Glutathione-S-transferases operates in cellular protection against oxidative stress, pollutans, and toxic external chemicals like heavy metals.
In our laboratory we detect polymorphisms of the most relevant metabolizing genes by real time PCR. We aim to individuate risk factors of dispositions to diseases before they appear, but also to enable and sustain a specific therapy for known diseases or intolerances.
In our new purpose we try to answer question of how we can use the predefined genetic difference in ability of detoxification, to explain why two persons, exposed to the same environmental factors, react in a different manner. Anyway we recommend genetic analysis and invite people to make use of them to preserve the own health.

CHARACTERIZATION AND SORTING OF FLOW CYTOMETRIC POPULATIONS OCCURRING IN HUMAN SEMEN

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Present tests for the diagnosis of male infertility are unable to reveal all the sperm traits that are needed to successfully reach the oocyte and properly deliver an intact paternal genome. Flow cytometry (FC) could accomplish this goal, thanks to its ability to simultaneously measure many parameters at the single cell level. Human semen may contain both somatic and germ cells, including immature elements other than spermatozoa. FC reflects the complexity of human semen in the linear FSC/SSC cytograms, where spermatozoa are contained within a flame shaped region (FR). In the latter, by simply staining nuclei in membrane permeabilized cells, further FC subpopulations can be observed: anucleated elements and two populations that differ by the nuclear labeling with propidium iodide (PI), termed $PI^{brighter}$ and PI^{dimmer} populations. In this study, we further characterized these semen FC populations. First, we verified whether FR does include all semen spermatozoa. We added fluorescent beads in PI stained sperm and then we calculated the ratio: nr sperm/nr beads by both light microscope, LM (r1) and FC within the FR (r2). We found that in all samples where the FR was clearly depicted, the values of r1 and r2 were similar, indicating that FR sperm overlap all semen sperm. Secondly, we demonstrated that PI^{dimmer} elements are actually spermatozoa by sorting PI stained semen samples by FACSAria II and observing them directly with LM. Finally, we studied the cause of the lower staining in PI^{dimmer} sperm, by hypothesizing that such sperm lost DNA fragments, due to a process of testis apoptosis interrupted during chromatin maturation. To investigate the presence of apoptosis and immaturity, we labeled sperm with FLICA and, in sorted PI^{dimmer} sperm, with aniline blue. We found that PI^{dimmer} sperm express signs of both immaturity (43.4±19.8%, n=5) and, above all, apoptosis (77.2±9.3%, n=9), suggesting that such population could derive by testis abortive apoptosis.

MECHANISMS ORIGINATING HUMAN SPERM DNA FRAGMENTATION

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Sperm DNA fragmentation (sDF) negatively impacts on human reproduction. The causes of sDF are not yet fully clarified, even if several mechanisms have been hypothesized. sDF could be due to an apoptotic process, as DNA cleavage is one of the late steps of this type of cell death. Another proposed mechanism is the failure in the re-ligation of those DNA nicks that are produced to favor the replacement of histones by protamines during sperm chromatin maturation. Finally, sDF could result from the

attack by reactive oxygen species (ROS), when the latter overwhelm the sperm antioxidant defenses. In this study we investigated these putative mechanisms of origin of sDF, using sperm sorting and tri-parametric analyses by the FACSAria instrument. The roles of apoptosis and oxidative stress were studied in sperm labeled by DAPI, TUNEL and, respectively, FLICA (a fluorescent inhibitor of caspases, CA) and a fluorescent secondary antibody revealing 8-hydroxy,2-deoxyguanosine (8-OHdG), the hallmark of oxidative DNA damage. To understand whether sperm with DF are immature, DAPI/TUNEL labeled semen samples were sorted to obtain sperm with and without DF, and the two fractions were stained with aniline blue (AB), that labels the excess of histones in sperm nuclei. Concerning the apoptotic pathway, we found that virtually all spermatozoa with DF express also the apoptotic enzymes (n=7). Conversely, the co-localization of 8-OHdG and sDF was found in a very small fraction of spermatozoa (3.5±2.0%, n=9). Finally, we found that in sperm with and without DF, AB labeling was respectively 76.5±18.6 and 37.2± 16.6 %, n=6 ($p<0.001$). This is the first report that directly investigated the causes of sDF. Apoptosis and, at a lesser extent, the impairment during chromatin maturation seem to be the main mechanisms responsible for sDF, whereas a direct role of ROS in inducing sDF before ejaculation appears to be marginal. However, ROS may indirectly induce sDF by triggering the apoptotic pathway.

SILVER NANOPARTICLES-INDUCED CHLOROPLASTS DAMAGE IN *LEMNA MINOR*: AUTOFLUORESCENCE SPECTROSCOPY AND IMAGING BASED ON CONFOCAL AND TWO-PHOTON LASER SCANNING MICROSCOPY

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Silver nanoparticles (AgNPs) are extensively applied as bactericides or fungicides. Moreover AgNPs are employed in merchandise like household appliances, cleaners, clothing, cutlery, children's toys, and coated electronics. As a result of this extensive application, a large fraction of the AgNPs commonly end up into aquatic ecosystems, where, due to their anti-bacterial properties, probably exert negative effects. In this study the toxicity of a suspension of AgNPs on the aquatic plant model *Lemna minor* (duckweed) was investigated. The effects of different concentrations of AgNPs (0.1-10 µg mL^{-1}) on the *Lemna* leaves were evaluated after 7-days exposure by analyzing autofluorescence spectra and optical imaging of *L. minor* via confocal and two-photon laser scanning microscopy. Fluorescence spectra were similar both in control and treated groups, but the fluorescence peak, around 685 nm, was less intense in treated plants. AgNPs-induced inhibition of autofluorescence in *Lemna* was dose-dependent. Furthermore, three-dimensional (3D) construction of the entire chloroplast structure by stacks of optical slices, shows that AgNPs-treated and control chloroplasts differ in thylakoid organization. Control chloroplasts display a fluorescence condensation with brightly appressed areas clearly separated by dark spaces, while thylakoid structure is completely disorganized in 10 µg mL^{-1} AgNPs -treated chloroplasts. Thus *Lemna minor* is a good biosensor for an accurate evaluation of environmental toxicity by metallic nanoparticles.

Hematology

CD45 SHIFTED EXPRESSION IN AN ATYPICAL CASE OF AGGRESSIVE NK LGL LEUKEMIA

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Background: Aggressive NK LGL (large granular lymphocytes) leukemia (ANKL) is a rare clonal lympho-proliferative disorder which represent less than 0.1% of all lymphoid neoplasm. ANKL is characterized by a systemic proliferation of NK cells that can involve both peripheral blood (PB) and bone marrow. Typically neoplastic cells show a CD45+, CD2-, sCD3-, cytCD3+, CD56+, CD57-, CD16+, phenotype with germ-line TCR genes. The disease course is typically fulminant with a very poor prognosis.
Aim: The aim of this study is to demonstrate the presence of a very rare population of NK cells with an atypical phenotype in the PB of a patient that two months later developed an ANKL.
Method: Two different flow cytometry analysis were performed in the PB of the same patient, respectively in January and March 2013. The antibodies panel includes: CD2, sCD3, CyCD3, CD5, CD7, CD4, CD8, CD16, CD56, CD57, CyTdT, CD158a and CD158b. Data acquisition and analysis were performed using a FACSCANTO II flow cytometer and FACSDIVA software.
Results. In January, a neoplastic NK cells population (0.3% of the total cells analyzed) was detected in the PB of the patient showing the following immunophenotype: sCD3-, CD7++, CD16+, CD56++, CD2-, CD5-, CD4-, CD8+dim, CyTdT-, CyCD3-, CD158a+, CD158b+. This rare cell population was aberrantly negative for CD45 antigen. Two months later, the leukemic cell population was increased to 23% of the total PB cells; the phenotype was concordant with the previously, except for the CD45 expression that was shifted to the positivity.
Conclusions. New diagnostic strategies need to be investigated in order to improve the outcome of ANKL patient. This ANKL case with an atypical and very rare CD45 negative immunophenotype at presentation can be useful to update the diagnosis management of this such aggressive disease.

MONITORING OF CHIMERISM ON SORTED PERIPHERAL CD34+ CELLS IN PATIENTS WITH ACUTE LEUKEMIA RECEIVING ALLOGENEIC BONE MARROW TRANSPLANT: A CASE REPORT

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Background. An early detection of neoplastic cells after allogeneic hematopoietic stem cells transplantation

(HSCT) in adult patients affected by acute leukemia provides the opportunity to manage and control the potential re-emergence of malignant clone. Several groups have reported as rapidly increasing mixed chimerism correlates with higher risk of relapse.
Aim. Some predictors of impending relapse are monitoring of Minimal Residual Disease (MRD) by flow cytometry (FC) and PCR-based chimerism on both Bone Marrow (BM) or Peripheral Blood (PB) samples at specific time-points after HSCT. Restricting chimerism analysis to sorted CD34+ cells could be an early predictor of relapse after HSCT.
Methods. At diagnosis was established for each patient the most useful leukemia-associated aberrant immunophenotype (LAIP) to be investigated at specific time-points during treatment plan to detect LAIP-positive cells. DNA was extracted from PB of donor and recipient before HSCT and chimerism assessment was performed on whole PB and sorted CD34+ cells of recipient after HSCT at specific time points: day 30, 60, 100, 180, month 12 and 18 from HSCT. The project is funded by *Ministero della Salute* and *Regione Toscana* (CUP D11J09000190003).
Results. The case we report on was enrolled in the project at April 2012. At day 30 from HSCT (matched unrelated donor), the chimerism on whole PB was 100% donor with MRD-negative; of note, chimerism on sorted CD34+ cells was 60% donor. At day 60, FC-MRD converted to positive (0.23% of global cells) whereas chimerism on whole PB was still 100% donor. At day 100, chimerism on whole PB decreased and the patient experienced BM relapse. Chimerism analysis on sorted CD34+ cells allowed a very early diagnosis of disease recurrence by highlighting the presence of the patient's cells at the first time-point with concomitant "negative signals" from FC-MRD and whole PB chimerism.

CD200 EXPRESSION IN MYELOID LEUKEMIC BLASTS

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INTRODUCTION. CD200 is a trans-membrane glycoprotein belonging to the type-1 immunoglobulin superfamily and is expressed on many different cell types including T and B lymphocytes and dendritic cells. The overexpression of CD200 has been implicated in the pathogenesis of solid tumors and hematological malignancies including lymphoma, myeloma, chronic lymphocytic leukemia and acute myeloid leukemia (AML).
AIM. Assess CD200 antigen expression on AML blasts and correlation of CD200 expression with AML subtype.
METHODS. The study included 44 patients, 21 male and 23 female, with a median age of 68 years (range 16-94). The FAB classification system divides our cohort of patients into six subgroups: 5, 6, 14, 5, 8, 6 subjects were respectively included in M0, M1, M2, M3, M4, M5 categories. Multiparameter flow cytometry analysis of bone marrow and peripheral blood was performed using a panel of monoclonal antibodies included CD200, CD13, CD33, CD34, CD14, CD117, HLA-DR, in order to asses antigen expression on AML blasts.
RESULTS. Blast cells of 21/44 patients (48%) expressed CD200. The expression of CD200 in the FAB subgroups was 5/5 in M0 (100%), 5/6 in M1 (83%), 5/14 in M2 (36%), 2/5 in M3 (40%), 4/8 in M4 (50%), 0/6 in M5 (0%). CD200 was co-expressed with staminality markers such as CD34 and HLA-DR. In 19 out of 21 patients CD200 was co-expressed with CD34 (CD34 was no expressed in the two CD200-positive M3). All CD200-positive samples except one (M3 patient) also resulted HLA-DR-positive. Only three subjects expressed CD200 and CD14 (monocytes marker). CD200 was co-expressed with CD13 and CD33 in 18/21 and 15/21 patients respectively. CONCLUSIONS. CD200 could be considered as a marker of myeloid blast cells less differentiated and introduced into the panel of immunophenotypic characterization of AML. Further studies are need to investigate the prognostic significance of this antigen.

SPONTANEOUS REGRESSION (SR) OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) TO MONOCLONAL B-CELL LYMPHOCYTOSIS (MBL): REPORT OF TWO CASES

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SR of CLL to MBL is rarely seen: 14 patients have been reported so far (Nahkla, 2013). We report herein 2 additional patients showing SR to MBL after the initial diagnosis of early stage CLL. The first patient was a 57-year old woman diagnosed with Rai stage 0 CLL in October, 1999. She had a white blood cell count (WBC) of $10x10^9$/L ($6x10^9$/L were CD19+CD20+CD23+CD5+ B-lymphocytes, with kappa light chain restriction). The patient was then managed by observation only; she maintained a stable lymphocyte count until December, 2006, when WBC count and differential were found normal (WBC $5.4x10^9$/L). However, a clonal B-cell population was showed ($2.4x10^9$/L). A normal WBC count was always found until the last evaluation (August, 2013), despite flow cytometry monitoring showed the persistence of a small population of clonal B-cells. The second patient was a 75-year old woman diagnosed with Rai 0 CLL in January, 2002. She had a WBC count of $10.6x10^9$/L ($7,1x10^9$/L were CD19+CD20+CD23+CD5+ B-lymphocytes, with kappa light chain restriction). The patient was followed-up with routine laboratory and clinical examination; she maintained a stable lymphocyte count until October 2009, when WBC count was found normal ($4.3x10^9$/L), with a mild lymphocytosis (45%) at differential count. From May, 2012 the patient maintained a normal WBC count until the last evaluation (August, 2013). Also in this case, periodical immunophenotyping showed the persistence of a small population of clonal B-cells. In both cases FISH analysis (for del13q14, del17p, del11q, trisomy 12) was negative, IgVH mutational status was mutated and either CD38 and ZAP-70 were negative. In conclusion, CLL is a neoplastic disorder with significant clinical and biological heterogeneity. The better knowledge of the factors able to induce SR or other types of regressions in this lympho-proliferative disorder, even in patients with advanced disease, could open the way to possible novel and targeted therapeutic strategies.

A SHORTER TIME TO THE FIRST TREATMENT(TFT) MAY BE PREDICTED BY THE ABSOLUTE NUMBER OF REGULATORY T-CELLS (TREGS) IN PATIENTS WITH RAI STAGE 0 CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Tregs are increased in CLL and correlates with clinical and biological features of active/progressive disease. We evaluated a cohort of 75 consecutive patients (pts) with Rai stage 0 CLL, in whom the absolute number of Tregs was determined at the time of diagnosis and correlated to main clinical and biological features, as well as to the need of receiving any specific therapy. Overall, 12 pts (16%) did reach a point during the follow-up (median time: 16 months) to need therapy. One pt was treated after just 2 months from diagnosis because of a sudden and relevant increase of lymphocyte count (from 105,400 to 189,200/μL). Three pts doubled lymphocytes after 5, 6, and 7 months, and then treated. The remaining 8 pts underwent therapy because of they shifted to a more advanced clinical stage, along with a relevant, progressive lymphocyte increase and appearance of clinical symptoms. None of these events occurred in the cohort of pts who did not receive therapy. Age, gender, and lactate dehydrogenase serum levels were not statistically different between untreated and treated pt groups ($p < 0.14$ and 0.36, respectively). Treated pts, however, showed a higher number of white blood cells and B-cell lymphocytes ($p < 0.001$), lower hemoglobin values ($p < 0.015$) and higher platelet count ($p < 0.012$). With respect to other biological prognostic factors, a greater number of cases with unmutated IgVH status ($p < 0.025$) and carrying high risk cytogenetic abnormalities ($P < 0.05$) was found in pts requiring therapy. By contrast, ZAP-70 and CD38 expression was not able to show any statistically significant difference between the two groups. Multivariate analysis confirmed that the absolute number of Tregs was an independent predictor of TFT in Rai Stage 0 CLL pts ($p < 0.001$). Finally, the best predictive cutoff of Tregs cells was 41/μL. These data show that the absolute Tregs cell number is able to identify Rai stage 0 CLL pts at higher risk of requiring therapy.

VALIDATION PROCESS OF STEM CELLS ENUMERATION/QUANTIFICATION: EXPERIENCE OF A SINGLE CENTRE

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Aim: In order to achieve JACIE accreditation was mandatory to validate the process of stem cells quantification/enumeration (ISHAGE Protocol). The study is retrospective and two years of transplant activity have been analyzed.
Method: ISHAGE Protocol (Stem Kit Enumeration –Becton Dickinson) was used; our laboratory participates to UK NEQAS external quality controls program. In this study were analyzed n. 165 patients undergone autologous transplant
Of each patient are taken into account: peripheral venous blood before harvest, apheresis product (HPC-A), the post-harvest peripheral venous blood and HPC-A infused. The entire route is on the same day. Of all types of samples are considered the following parameters: viable TNC viable %, (7-AAD), viable MNC % (7-AAD), viable CD34 % (7-AAD), enumeration/quantification of CD34+ (absolute value and percentage), MNC % in the HPC-A.
Results: With regard to peripheral venous blood pre-harvest: TNC is 99%; MNC is 100%; CD34+ is 98.6%; enumeration/quantification of CD34+ cells is 137 (cells/ul) and 12:31% (total population).
With regard to peripheral venous blood post-harvest: the TNC is 98.6,%; MNC is 100%; CD34+ is 98.6%; enumeration/quantification of CD34+ cells is 61 (cells/ul) and 0.2% (total population).
With regard to HPC-A: TNC is 98.2%; MNC is 99.7%; CD34+ is 97.8%, the enumeration/quantification CD34 + is 2134 (cells/ul) and 1.94% (total population).
With regard to HPC-A infused: TNC is 66.1%; MNC is 88.7%; CD34+ is 68.3%, enumeration/quantification of CD34 + is 1401 (cells/ul) and 1.52% (total population).
The values are expressed in terms of the median.
Conclusions: The flow-cytometry performance for the validation of the results required at the end of a HPC collection is considered good: there was no "graft failure" and engraftment were in due time.

ROLE OF CIRCULATING αβ-DOUBLE NEGATIVE T CELLS (DNT) IN LYMPHOMA PATIENTS: PRELIMINARY RESULTS OF A PROSPECTIVE STUDY

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Many aspects of lymphoma pathophysiology indicate mutual interactions between the host immune system and lymphoma cells. An unconventional subset of CD4−CD8− double-negative T cells (DNTs) has been described to specifically contribute to anti-tumor immunity, acting as both regulatory T cells and/or cytotoxic T cells. No data are available on their role in human anti-lymphoma immunity, their interaction with other immune cells, functional attitude and prognostic significance in lymphomas.
The aim of study is to assess the frequency and the functional attitude of circulating DNTs in Lymphoma patients (pts) in order to evaluate their predictive role on clinical outcome.
Peripheral blood of 30 Lymphoma pts and 16 healthy donors were prospectively collected for phenotypic and functional characterization of DNTs by staining with: CD3,CD4,CD8,CD56,CD45,TCRαβ,CD45Ra,CD45Ro,CCR7,CD27,CD28,CD30,CD69,GITR,CD95,CD178,CD152,IFN-γ,TNF-α,granzymeB,perforin.For functional studies, DNTs were purified from PBMCs of pts by immune magnetic selections. Data were acquired using a 8-colour flow cytometer.
We observed a significant decrease ($p = 0.006$) of αβ-DNTs in the PB of pts with untreated lymphoma (20.5 ± 4.8 SE,) as compared with healthy controls (31.3 ± 3.4),and their number correlated with disease relapse/progression. In Hodgkin's Lymphoma pts the αβ-DNTs frequencies were significantly increased as com-

pared with other histotypes.Furthermore in ex-vivo expanded DNTs was observed a characteristic cytokines profile with a cytotoxic potential mainly characterized by the increase of IFN–γ and granzyme B secretion.
Our study has demonstrated for the first time that αβ-DNTs may play an important role in both the development and the progression of lymphomas. In addition, in our preliminary results, it is likely that ex-vivo expanded DNTs exert an anti-tumor activity, thus suggesting their possible use as new strategy for adoptive immune-therapy.

PERIPHERAL BLOOD MONOCLONAL T LYMPHOCYTOSIS AND RISK OF T LYMPHOPROLIFERATIVE DISEASE DEVELOPMENT

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Background: Occasional evidence of monoclonal gammopathy of undetermined significance (MGUS) or monoclonal B lymphocytosis (MBL) are considered predisposing to the development of multiple myeloma and chronic lymphocytic leukemia (CLL) respectively and it is recommended to monitor them for possible early diagnosis. In cutaneous T lymphoma (CTL), such as mycosis fungoides and Sezary syndrome, has been demonstrated the presence of clonal T lymphocytes even in the peripheral blood.
Aims**:** It seems possible that the detection of asymptomatic clonal proliferations lymphocytosis attributable to CTL may represent a predisposing condition to the development of cutaneous lymphomas.
Methods: Every year we analyze by flow cytometry about 1700 samples. Patients with peripheral lymphocytosis undergo a standard panel for T, B and NK cells. If clonal B is excluded, we proceed to phenotypic T CD4 subsets (CD7, CD26), analysis of V chains and TCR when necessary.
Results: From January 2010 to September 2012 we observed 28 cases of CD3 T> 80% of total lymphocytes (CD3 = 3.000/mm3). Lymphocytosis was variable between 3,000 and 10.000/mm3, with an average of 5,350. 14 subjects with CD4+CD8- > 75% of T cells showed CD7 low/neg, CD26 low/neg- and TCR V suggestive of monoclonality, then confirmed by molecular biology. These subjects were followed up with blood count and clinical / dermatological evaluation
twice a year in the event of increased peripheral lymphocytosis or appearance of symptoms suggestive of lymphoma. Two patients developed erythematous skin lesions and skin biopsy diagnosed the presence of Sezary syndrome, also confirmed on lymph node and bone marrow biopsies. Both these patients belonged to the subgroup with CD3 + CD4 + CD7-/ + CD8-CD26- phenotype, typical of Sezary syndrome.
Summary / Conclusion: 7.1% (2/28) of patients with monoclonal T lymphocytosis have developed a Sezary syndrome in a median follow-up of 20 months. Based on these preliminary data the occasional finding of T lymphocytosis with “atypical” phenotype CD3 + CD4 + CD7- / + CD26-, may be a useful tool in order to allow early diagnosis of CTL in analogy with the strategy currently used for patients with MBL. If the hypothesis will be confirmed in larger cohort this approach could represent an advantage in terms of early diagnosis and timely treatment in this subset of patients.

PROANGIOGENIC EFFECT OF BONE MARROW FIBROBLASTS IN PATIENTS WITH MULTIPLE MYELOMA

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Bone marrow (BM) stromal cells favor progression of multiple myeloma (MM) but cancer-associated fibroblasts (CAFs) have not been studied yet. CAFs were increased in patients with active MM (at diagnosis, at relapse) compared to those in remission, those with monoclonal gammopathy of undetermined significance (MGUS), and those with deficiency anemia. They displayed an activated phenotype, and produced high levels of TGFβ, IL-6, SDF1α, and IGF1. They showed a heterogeneous phenotype which entailed their origin from resident fibroblasts, and from endothelial cells (ECs) and hematopoietic stem and progenitor cells via endothelial-mesenchymal transition, and from mesenchymal stem cells via mesenchymal transition, all induced by CAFs themselves and MM cells. Active MM CAFs induce proliferation and apoptosis-resistance of MM cells through cytokines and cell-to-cell contact. Studies in syngeneic 5T33MM and xenografted mouse models showed that MM cells induced the CAFs expansion which, in turn, favored MM initiation and progression as well as angiogenesis. *In vivo* Matrigel plug assays and *in vitro* chemotaxis showed the ability of active MM CAFs (as cells and CM) to attract mouse and human $CD31^+$ ECs, and have a direct angiogenic effect. They support MM tumor growth by promoting its neovascularization and contribute to the angiogenic switch and the subsequent angiogenic phase which parallel transition of MGUS into MM. Moreover in patients’ and mice BM biopsies nests of CAFs were found in close contact with MM cells suggesting a vascular protective niche. Targeting CAFs in MM patients may be envisaged as a therapeutic strategy.

COMPARISON OF DIFFERENT PLATFORMS IN FLOW CYTOMETRY FOR ENUMERATION OF RESIDUAL LEUCOCYTES IN LEUCO-DEPLETED BLOOD COMPONENTS

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A reliable method to count low levels of white blood cells (WBCs) in platelet concentrates (PCs) and leucocyte-depleted red cell concentrates (RCCs) is essential for Quality Assessment of these leuko-depleted products. A comparison was performed using two different counting methods. The first method was based on pre-counted fluorescent beads (FlowCount® beads) and was performed on two flow cytometer analyzers that introduce sample by a pressure differential. The second was based on a volumetric device (Partec CyFlow® Cube6) whereby an analysis and count is performed on a fixed volume (as defined by the distance between two platinum electrodes) or a desired volume may be freely selected by software.
For both methods, PC and RCC samples were labeled with a mixture containing a membrane permeabilizing

agent, RNAse and propidium iodide to discriminate the nucleated cells. WBC absolute numbers obtained with the addition of beads were derived from the ratio of cells to beads. True volumetric absolute counting (TVAC) of WBC's was performed with the addition of 500uL of PC or RCC sample prepared with a mixture provided by Partec to the Cube6 Instrument. A fixed volume of sample (200uL and/or 800uL) was determined automatically through the passage of the sample between two electrodes or determined by software. WBC numbers were obtained directly and reported as WBCs/mL as displayed at the end of acquisition.
Prior to experimentation, peripheral blood from a healthy donor was used to set up the appropriate gating strategy on the different instruments.
There was a positive correlation in the number of leucocyte counts between the compared counting methods and instrumentation. We also found a good correlation (r > 0.90) between the two counting methods tested on the volumetric device (electrodes and software control).
In conclusion, the Cube6 instrument provides an alternative rapid and reliable method for counting very low numbers of leucocytes in leuko-depleted products.

A IMMUNOPHENOTYPIC VARIANT HAIRY CELL LEUKEMIA. ROLE OF THE CYTOMETRY

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Hairy cell leukemia(HCL) is a mature B-cell non-Hogkin lymphoma characterized by pancytopenia, splenomegaly(96% of cases), lymphadenopathy (35% of cases) and an indolent clinical course. Diagnosis is based on morphology, bone marrow biopsy(BM) and flow cytometry (FC). The treatment of choice is purin nucleosid analogues which overall responses in >85% of patients. Rituximab has been recently introduced for treatment of HCL patients prolonging event free survival. We report the case of a woman (75 years old) with severe pancytopenia and splenomegaly. The BM biopsy showed an HCL with an unusual immunophenotypic profile (IIC: CD20+; 4 KB5+; CD3-; CD5-; CD23+; CD25+; CD10+; CD43+/-; BCL6-), therefore FC of peripheral blood was requested. Cells were stained with CD3, CD4, CD5, CD8, CD10, CD19, CD20, CD22, CD23, FMC7, CD103, CD11c, CD25, CD38, CD45, κ and λ mAbs and analyzed with a FACS Canto flow cytometer with Diva software. The analysis showed an infiltrate (5%) of cells with a dim Side Scatter positive for CD103, CD19, CD20, CD22. The 50% of these cells were positive for CD25, CD11c, CD10 and CD23, showing an atypical phenotype. The patient started therapy with pentostatin without benefit as pancytopenia and splenomegaly persisted. BM showed the same of the onset. Therefore therapy with Rituximab, 375mg/m^2 for 4 cycles, was introduced. Two months after the end of therapy, the blood count improved and splenomegaly was reduced. Therefore, the patient didn't obtained response to purine analogues therapy, instead she obtained response to anti- CD20 therapy. In conclusion, FC is a tool with a great diagnostic power and a low cost and it is an integral component of contemporary hematopathology practice. We hypothesize that the forms of HCLs, who don't respond to therapy, need a different approach of the clinicians on the use of appropriate therapeutic protocol that can be suggested by the immunophenotypic analysis.

THE IMMUNOPHENOTYPING CAN BE A PROGNOSTIC FACTOR IN ACUTE PROMYELOCYTIC LEUKEMIA

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Acute Promyelocytic Leukemia(APL) is a rare disease characterized by chromosomal translocation t(15;17) leading to fusion gene PML/RARα that responses to maturation inducing treatment with all trans-retinoic acid(ATRA). Flow cytometry(FC) mat improve the accuracy of diagnosis and leads to detection of APL forms with worse prognosis. We present a distinct subtype of APL as defined by FC analysis, in a young woman with leukocytosis, anaemia and thrombocytopenia, cutaneous hemorrages and low fibrinogen level. The bone marrow smears revealed an infiltrate of big granulated blasts. Leukocyte cell suspension from peripheral blood(PB) and bone marrow(BM) were stained with CD2, CD3, CD4, CD7, CD8, CD10, CD11b, CD19, CD20, CD22, CD13, CD14, CD15, CD16, CD33, CD34, CD64, CD56, CD117, HLA-DR, CD45 mAbs and analysed by FC using a FACS Canto flow cytometer with Diva software. The analysis of PB and BM revealed an infiltrate of immature myeloid cells(84%) CD64$^+$, CD33$^+$, CD13$^+$, CD2$^+$, CD117$^-$. Cells had an high side scatter like a classic APL form and 16% of these blast cells were CD34$^+$. Patient started AIDA protocol and biomolecular analysis confirmed gene fusion PML/RARα positive after 24 hours. On day 4 patient had side effects related to ATRA Syndrome and Pseudotumor cerebri(PC). ATRA was discontinuated and Dexametasone and Furosemide therapy was promptly started. On day 6 patient died for intracranial haemorrhage. The blast cells had a pattern of an atypic APL CD34 positive and CD117 negative surface markers. Moreover, blast cells coexpressed the T lineage CD2 with myeloblastic marker typic of the hypogranular variant form. We ipotize that immunophenotipic analysis may be useful for risk stratification in APL patients, allowing to identify patients with bad prognosis and a major risk of bleeding, ATRA syndrome or PC. It is necessary to analyse a large number of cases to include the immunophenotype as individual prognostic factor in APL patients.

CHARACTERIZATION OF BAFF-RECEPTOR EXPRESSION IN LEUKEMIC LYMPHOID/MYELOID CELL LINES AND IN PEDIATRIC B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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B cell-activating factor (BAFF) and its close relative proliferation-inducing ligand (APRIL) belong to the tumor necrosis factor (TNF) family, which mediate the behavior of most B cells and through interactions with their family receptors. Among them, only the BAFF receptor (BAFF-R) interacts specifically with BAFF, being the main responsible for primary B cell survival, selection and dif-

ferentiation, in physiology and in disease conditions.
We aimed to investigate the potential role of BAFF/BAFF-R axis in Acute Lymphoblastic Leukemia involving B-Cell Precursor (BCP-ALL). With this purpose, we analyzed the pathway in hematological tumor cell lines well as in primary BM and PB samples from children affected by BCP-ALL.
Preliminary results demonstrated that BAFF-R is highly expressed in the B-lymphoid leukemic cell lines, such as REH, TOM1 and NALM-6 (RT-PCR analyses). Its expression is also detectable, although at lower levels, in mixed lymphoid/myeloid phenotype cell lines (such as THP1 and RS4;11), in myeloid K562 cells as well as in U937 histiocytic lymphoma cell lines. Supported by this data, we further collected BM and/or PB of 24 consecutive diagnostic samplesof Pediatric BCP-ALL. We analyzed BAFF-R expression by flow cytometry, byindirect staining using a biotinylated antibody anti-BAFF-R (kindly provided by Prof. Rolink, UniBas, Switzerland) and anti-streptavidin-PE as a secondary antibody. In the same sample we assessed the CD19 (FITC), CD10 (APC) and CD45 (PerCP) direct staining to recognize leukemic blast cells among the residual of normal cells. For each patient, in addition to the diagnostic sample, we analyzed at least one follow up sample (i.e. at day+8, +15 or +33). We detected high levels of BAFF-R on CD19+CD10+CD45dim leukemic cells, which persisted during the follow up treatment. Moreover, we separated plasma by centrifugation of n=14 patients to analyze BAFF cytokine levels by ELISA technique, and we observed higher levels at diagnosis compared to follow up samples.
In conclusion, we assessed the expression of BAFF-R on leukemic blast cells of BCP-ALL patients. Of note, its expression is maintained on residual tumor cells during the drug treatment, suggesting the potential targeting of this molecule in future advanced treatment approaches. All together, BAFF/BAFF-R axis could have a role in B-ALL, although additional studies are required to comprehend its role in the pathogenesis of leukemia.

THE INTEGRATION OF: MULTIPARAMETRIC FLOW CYTOMETRY, AUTOMATED CYTOMETRY AND MORPHOLOGY IN THE CHARACTERIZATION OF A CASE OF MASTOCYTOSIS

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Objectives: To explain the rapid variation of the cytogram in a patient in follow-up for MDS after splenectomy.
Methods: Woman, 81years, followed by 4 for MDS, in the E.R. because of fatigue, fever and abdominal pain. On examination: splenomegaly, for which were performed further investigations: CBC made by SIEMENS ADVIA 2120i, immunophenotypic characterization with Cytomic FC500 B & C, evaluation of peripheral smear.
Results and conclusions: CBC, compared with previous, showed a worsening of anemia and thrombocytopenia. Since the patient was known for MDS (RCMD), was not made morphological evaluation in urgency. Meanwhile, the CT scan confirmed splenomegaly, with the presence of two nodules for which was practiced splenectomy. The following CBC was completely altered compared to the previous, showing marked increase in LUC and pseudobasofilia. Smear showed cellular elements medium/ large, oval / elongated, with thin cytoplasmic ramifications, round-oval or bilobed nucleus covered with large red-black granules: the morphology suggested the presence of mast cells. Dissolution was immunophenotype which showed a population with increased complexity (SSC), positive for CD33, CD117, CD38, CD2, CD25 and negative for Ag of immaturity / basophils. Positivity for CD2/CD25 likely due to the negative elements for peroxidase and morphologically similar to basophils, suggested the presence of atypical mast cells. All confirmed by biopsy. In conclusion, although the CBC at entrance in E.R. was compatible with a progression of MDS, the rapid change of cytographic feature after splenectomy, suggested the presence of "atypical" cell populations that required careful characterization. Given the complexity of the morphology and the low prevalence of mast cell diseases, decisive was contribution of flow cytometry in the correct diagnosis of the disease.

INTEGRATING POST INDUCTION WT1 QUANTIFICATION AND FLOW CYTOMETRY RESULTS IMPROVES PROGNOSTIC STRATIFICATION IN ACUTE MYELOID LEUKEMIA (AML)

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Detection of minimal residual disease (MRD) has a relevant prognostic value in AML. In the present retrospective study we evaluated fifty uniformly treated adult non M3 AML patients with respect to pretreatment risk factors and post induction MRD analysis. Forty-two patients (84%) achieving CR after first induction regimen were evaluated for reduction of WT1 gene expression (ΔWT1=logWT1 at diagnosis –log WT1 post induction); 34 patients for residual leukemic cells by flow cytometry (flow-MRD) (≥2,5 x 10^{-4} residual leukemic cells defined positive samples). Twenty-three patients (68%) were flow-MRD^{pos}, 11 (32%) were flow-MRD^{neg}. The post induction residual clonal population detected by flow had a strong prognostic value: 3-year DFS was 79,5% and 27,3% in flow-MRD^{neg} and in flow-MRD^{pos} patients respectively (p =0.032). None of the patients older than 60 years of age reached a negative flow-MRD, while among 14 high risk profile patients only one was flow-MRD^{neg} but relapsed after BMT. In the 23 flow-MRD^{pos} patients, DFS was not affected by the amount of residual neoplastic population, above or below 1% (p=0.41). Flow-MRD^{pos} patients were stratified according to kinetics of WT1 reduction. Patients (13) with a ΔWT1≥1.5 log had a better outcome compared to those (10) with ΔWT1<1.5 log (3-year-DFS was 46.2% vs 0%, p<0,001). High risk profile patients at diagnosis and patients not achieving ΔWT1≥1.5 log post-induction form unique poor risk subgroups of patients that should receive alternative treatments including BMT.

MULTI-LINEAGE DYSPLASIA AS ASSESSED BY IMMUNO-PHENOTYPE HAS NO IMPACT ON CLINICAL-BIOLOGIC FEATURES AND OUTCOME OF ACUTE MYELOID LEUKEMIA WITH MUTATED NUCLEOPHOSMIN (NPM1)

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Background. Acute myeloid leukemia (AML) with multilineage dysplasia (MLD) is a separate subset in WHO classification. Morphologic study of residual hemopoiesis is the standard criteria for defining MLD. Its prognostic value is still under debate: i) morphology is operator-dependant; ii) MLD-related poor prognosis is supposed to rely on progression from a myelodysplastic syndrome (MDS) but MLD might merely result from maturation by AML clone. A major controversy concerns MLD value in NPM1-mutated (NPM1+) AML, since NPM1+ status correlates with good prognosis. Aims. To study MLD in NPM1+ AML by an alternative technique: flow cytometry (FC) is emerging for studying dysplasia. Its application to AML allows: i) to study large cellsí amount; ii) to quantify, refer to controls and standardize FC parameters. Methods. Patients: 70 NPM1+ pts were studied. Flow cytometry: FACSCanto II (BD) and Infinicyt (Cytognos) were used for acquisition and analysis. MLD was appraised by an immuno-phenotypic score (IPS) including 18 parameters (14 for granulocytic and 4 for erythroid lines). Results. Median age was 57 (24-70); WBC count was 48.0 x 109/L (1.2-260.0). Karyotype was normal in 62 (88.6%) pts. FLT3-ITD occurred in 27 pts (38.6%). IPS was calculated in all 70 pts; median IPS was 6.25 (0.5-18.5). Pts were grouped according to IPS higher (IPS+) or lower-equal (IPS-) than the median: age, WBC, platelets, morphologic MLD were not different. IPS+ group had lower FLT3-ITD incidence (25.7% vs 51.4%; p=0.048); interestingly, Falini et al (Blood 2010) reported analogue results by morphology. CR rate was not different in IPS- (82.9%) and IPS+ (74.3%; p=0.56) pts. IPS did not affect disease-free and overall survival. Conclusions. This study provides evidence that MLD, as assessed by FC, does not influence clinical features and outcome of NPM1+ AML. These findings support NPM1+ AML to be considered as a separate entity and its prognostic assessment should not be based on MLD.

POST-INDUCTION MRD EVALUATION: A COMPARISON OF MULTIPARAMETER FLOW CYTOMETRY AND WT1 RT-PCR TECHNIQUES IN 51 ACUTE MYELOID LEUKEMIA (AML) PATIENTS

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We evaluated post-induction bone marrow mimimal residual disease (MRD) in 51 AML adults, with median age of 57 years (range: 22-76). MRD was measured both by multiparameter flow cytometry (MPFC) and by WT1 expression, by RT-PCR. Cytometry tests were performed in 43pts, cytogenetics status was known in 48 pts; NPM and FLT3 status were known in 50pts, defining the molecular-cytogenetic risk in 43pts: 18 favorable, 19 intermediate1, 6 unfavorable. With a median follow-up of 12 months (range 2-49), CR rate after induction therapy was 84% and Relapse Free Survival (RFS) was 82%; according to cytogenetics and molecular markers, CR rate was 100% in favorable cytogenetic, 86% in NPM+FLT3-, 69% in double negative, 60% in FLT3+, 40% in unfavorable cytogenetics. WT1 was +ve in 36/48 pts (75%) at diagnosis (median 1155; 51.9-9176) and all pts showed >20% blasts by MPFC. Post induction WT1 was +ve in 4/39 (10%) (median 19.6; 0,6-13463.3) and by MPFC MRD was +ve in 10/36 (28%) pts.MRD evaluated by MPFC and WT1 was concordant in 27/33 (82%) pts after induction. We analyzed the predictive value of post induction MRD (evaluated by MPFC and WT1) in terms of RFS and OS. In pts with†WT1 -ve (35/39: 90%) OS was 69% (median 20 months; 2-58); in 4/39 (25%) with†WT1 +ve OS was 25% (median 7 months; 5-34); MPFC was -ve in 26/36 (72%): in these pts OS was 77% (median28 months; 2-58); MPFC was +ve in 10/36 (28%): OS was 30% (median7 months; 3-34). We observed 9 relapses. Among pts with WT1 -ve RFS was 86% (median22 months; 1-57), while in WT1 +ve RFS was 50% (median19 months; 5-33). Among pts with MPFC -ve RFS was 96% (median 27 months; 2-48), while in MPFC +ve RFS was 50% (median 4 months; 1-33). In conclusion, our data suggest that WT1 and MPFC are complementary tools for MRD evaluation. Both the techniques were concordant in 82% of cases, but MPFC predicts better thanWT1 the outcome (in terms of OS and RFS respectively). These data should be confirmed in a prospective study.

EVALUATION OF BONE MARROW DYSPLASIA BY FLOW CYTOMETRIC ANALYSIS WITH ACCEPTABLE INTER-OPERATOR VARIABILITY: A PROSPECTIVE MULTICENTRIC STUDY

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The assessment of marrow dysplasia is the mainstay for the diagnosis of myelodysplastic syndromes (MDS), and represents the basis of the WHO classification of these disorders. We conducted a prospective multicentric study to evaluate the clinical utility of immunophenotyping in the diagnostic and prognostic work up of MDS patients.
We first studied a ëílearningíí cohort of 191 patients whose analysis was aimed at defining the set of cytometric variables with a different expression between MDS and controls affected with non-clonal cytopenia. Analysis of myeloid cells showed increased prevalence of abnormalities on CD16/CD13 and a CD16/CD11b maturation

pattern (P<.001), reduced granulocytic SSC (granulocytes to lymphocytes SSC peak channel ratio, p<.001) and lower proportions of CD10+ granulocytes (P <.001) in MDS. Analysis of CD34+ cell compartment showed increased CD45+CD34+ myeloblasts (P<.001), reduced CD34+ B-cell precursors (P<.001), increased expression of lineage infidelity markers (CD5, CD7, CD19, or CD56, P<.001), and abnormal CD45 expression (lymphocytes to myeloblasts CD45 ratio, P<.001). Analysis of erythroid cells showed decreased levels of CD71 (P<.001) and increased expression on cytosolic ferritin (P<.001) on nucleated red cells.
The weight in the recognition of bone marrow dysplasia of each parameter that matched criteria of adequate inter-operator reproducibility (i.e., coefficient of variation <20%) was tested by a multivariable general logistic regression model. A flow cytometric score (FCM-score) for the diagnosis of MDS was defined based on regression coefficients from that model. A score value of 1 was assigned to CD34+ myeloblasts >1.5%, B cell progenitors <4.8% in all CD34+ cells, lymphocytes to myeloblasts CD45 ratio <4.1 or >7.8, granulocytes to lymphocytes SCC ratio <6.3, CD10+ granulocytes <28%, CD71 MFI expression on erythroblasts <164, while a score value of 2 was assigned to CD34+ myeloblasts >3.5%. A diagnosis of MDS was formulated in presence of a FCM-score value >2
We assessed the diagnostic power of FCM-score in the ëílearningíí cohort. Overall 115/128 MDS patients received a correct diagnosis (sensitivity 90%). Five false-positive cases were noticed among 63 controls (specificity 92%). In MDS patients, a high score value (>5) was found to be associated with the presence of multilineage dysplasia (P=.001), excess blast (P<.001), transfusion-dependency (P=.03) and poor risk-cytogenetic (P=.04), resulting in a higher IPSS risk (P=<.001). An independent validation of the obtained FCM-score was carried out on a series of 255 patients (ëívalidation cohortíí). The obtained sensitivity and specificity were 89% and 91%, respectively.
Finally we evaluated the prognostic effect of the FCM-score in 213 patients with a conclusive diagnosis of MDS and complete survival information. In multivariable analysis together with demographic factors and IPSS risk, the FCM-score significantly affected the probability of overall and leukemia-free survival (HR 1.91 and HR 2.69, P<.001).
These results indicate that immunophenotyping based on parameters with acceptable inter-observer reproducibility may help to establish the diagnosis of MDS. Moreover, the evaluation of marrow dysplasia by flow cytometry may have important prognostic implications

B MEMORY COMPARTMENT AND CD38+ PLASMA CELLS IN MULTIPLE MYELOMA PATIENTS

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Introduction: Multiple Myeloma (MM) is characterized by monoclonal plasma cells accumulation in bone marrow. Recently cancer stem cells (CSC) are attracting attention. In MM peripheral blood (PB) and bone marrow (BM) samples, CD138- B cells sharing same molecular alterations with MM plasma cells were identified. These cells show properties of stem cells (side population) and memory B cells. (Matsui et al. Cancer Res 2008). Given that, we studied B memory compartment.
Material and Methods: Thirty-five MM BM and PB samples were analyzed using the following panels: 1) CD24 FITC/ IgD PE / CD45 PercP-Cy5.5/ CD38+CD138 APC/CD20 APC-H7/CD19 Pacific Blue; 2) CD27 FITC/ CD56 PE/ CD45 PercP-Cy5.5/ CD38+CD138 APC/CD20 APC-H7/CD19 Pacific Blue. Almost 1000 on CD19 gate and CD38+138+ were acquired. Acquisition and data analysis were performed with CyAN ADPTM and Kaluza 1.1 (Beckman Coulter, Miami, FL, USA) respectively. WBC count on PB and BM was performed with DXH800 (Beckman Coulter, Miami, FL, USA).
Results: Naïve B cells are the most representative population in PB (M= 57.5%) and BM (M=51.35%). Late memory cells are less represented in PB (M=21.93%) than in BM (M=27.67%). In both PB and BM, according to the Bm1-Bm5 classification, B late memory cells are distributed between the gates identifying Bm5 early (BM=7.5%; PB=7%) and Bm5 late (BM=24.2%; PB=24.9%) cells; naïve B cells are included in Bm1 (BM=30.4%; PB=32.6%) and Bm2 (BM=20.23%; PB=24.9%) gates, on the CD38-CD138/IgD dot plot.
Conclusions: This study has provided an elegant starting point for analysis of CD19+ B lymphocytes antigen-dependent maturation in MM BM and PB, and relationship with pathological plasma cells.

USE OF CELL POSITIONAL DATA AND MULTIPARAMETER FLOW CYTOMETRY TO IDENTIFY B CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

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Objectives: B cell chronic lymphocytic leukaemia (B-CLL) is a clinical-biological heterogeneous disease. Coulter automated laboratory analyzers differentiate WBC based by measures of their volume, conductivity and scatter. We investigate the predictive value of index measures, known as cell population data, compared to morphologic and flow cytometric analysis for detection of chronic lymphocytic leukemia (CLL).
Methods: Morphology, blood cell counts, flow cytometry and cytogenetics analysis were performed in samples from 35 patients with B-CLL and 84 healthy using the Beckman Coulter LH780 and DXH800 and the cytometer Cyan ADP TM †(Beckman Coulter, Miami, FL, USA). Volume, Conductivity, Scatter, and antigen distribution patterns of lymphocytes compared to cytogenetics were evaluated as predictors of disease
Results: B-CLL immunophenotype patterns showed CD5+CD23+ homogeneous /spread and CD5+CD23+ spread/spread and mean of lymphocytes volume showed most relevant difference in comparison to healthy samples (p = 0,0001), the area under the ROC curve (AUC) =0,897. No difference appeared in conductivity (mean ±SD) and in light scatter (mean±SD) values. Dividing patients into cytogenetic subgroups according to interphase fluorescent in situ hybridization (i-FISH) data, a difference emerged in mean of lymphocyte volume among patients carrying del13 and all the others cytogenetic groups: the del13 group showed a lower mean of lymphocyte volume in comparison with other genetic group.
Conclusions: cell population data and immunophenotype pattern could be routinely used to screen for CLL and to integrate cytogenetic data.

EPIDEMIOLOGY OF MATURE B CELL NEOPLASMS IN THE TERNI AREA FROM 2010 TO 2012 USING FLOW CITOMETRY

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We performed a preliminary study to asses the incidence of mature B cell neoplasms CD5+ and CD5- in the Terni area in order to later evaluate the impact of environmental contaminants on the incidence and prevalence of such tumors. We used FACS Canto II BD and the following antibody panels in Peripheral Blood (PB) samples: for the routine panel CD3 FITC, CD16PE, CD45PerCP-Cy5, CD4PE-Cy7, CD19APC, CD8APC-Cy7; for the mature B cell neoplasm panel CD5FITC, CD10PE, CD19PerCP-Cy5, FMC7 FITC,CD23PE, CD38 FITC, CD56PE, CD20APC-Cy7, CD22APC, CD25PE-Cy7, CD11cAPC. Within the population studied, the mature B cell neoplasms are by far the most frequent findings. This is in agreement with WHO classification of haematopoietic and lymphoid tissues. We diagnosed 160 new cases of mature B cell neoplasms during the 3 years studied (2010-2012) with an incidence 14, 16 and 17 mature B cell CD5+ neoplasms /100,000 inhabitants in the year 2010, 2011, 2012 respectively. In particular CLL went from 7.7 in 2010 to 10 cases/100,000 inhabitants in 2011 with a change in man vs woman ratio (2:1 in 2010 to 1:1 in 2011) and a peak of incidence in the population of > 70 years old in the studied period. The most frequent phenotypes of CLL were CD20+dim CD22+ CD23+ and Ig k or lambda light chain dim. Mature B cell neoplasms CD5- instead decreased from 7.2-7.2-6.1/100,000 inhabitants in 2010, 2011and 2012 respectively. The most frequent CD5- B cell neoplastic phenotype were FMC7+, CD20+, CD22+ and CD20+,CD22+. In addition, we found 20 new cases of atypic CLL as defined by the WHO classification from 2010 to 2012.

PROGNOSTIC IMPACT OF MINIMAL RESIDUAL DISEASE ASSESSED BY MULTIPARAMETER FLOW CYTOMETRY IN CHILDHOOD ACUTE MYELOID LEUKEMIA

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Background: Achievement of morphologic complete remission (CR) is a prerequisite for cure in acute myeloid leukemia (AML), but patients in CR may still have variable levels of occult leukemia, defined as minimal residual disease (MRD). More sensitive methods are required to allow detection of leukemic cells: multiparameter flow-cytometry (MFC) is based on the research of aberrant expression of surface antigens on the malignant cells, to identify residual cells not detectable by morphologic assessment.
Aim: Aim of this study is to evaluate the prognostic impact of MFC-MRD in children with AML.
Materials and Methods: Patients younger than 18 years affected by de novo AML (diagnosis May 2003- May 2011), enrolled in AIEOP-LAM 2002 protocol, were eligible for this study.
Bone marrow aspirates from 142 patients were collected at diagnosis, at the end of induction 1 (ICE1) and part at the end of induction 2 (ICE2).
MRD was assessed during follow-up, by five-color MFC, through the detection of patient-specific leukaemia associated aberrant phenotypes (LAIPs), defined for each patient at diagnosis.
Results: Among the 142 patients recruited to this study, at the end of ICE1 35,9% had a level of MRDe"1%, 11,3% with MRD 0.1-1% and 48,6% with MRD<0,1%; 6 cases were not evaluable. We assessed the correlation between levels of MFC-MRD and disease-free-survival (DFS), by means of the Kaplan-Meier method. Presence of MRD e" 0,1%, at the end of induction 1, was associated with a DFS of 35,29 % + 7,23 % at 6 years, whereas MRD < 0,1% was associated with a DFS of 73,16+ 5,62% at 6 years ($p < 0,01$). Similar DFS data were obtained at the end of ICE2 ($p < 0,01$).
Conclusions: Our study revealed a significant correlation between MFC-MRD at the end of induction 1, and the patient's outcome: MRD≥ 0,1% appeared to be predictive of poor outcome. Our data suggest that levels of MFC-MRD at the end of ICE1 provide important prognostic information that may be used to a better stratification and to guide therapy of pediatric patients with AML.

EXPANSION OF CIRCULATING NATURAL KILLER POPULATION IN MULTIPLE MYELOMA PATIENTS AFTER LONG-LASTING THERAPY WITH LOW DOSE LENALIDOMIDE

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Effects of lenalidomide (LD-R) in Multiple Myeloma (MM) patients are to its direct anti-proliferative and anti-angiogenic properties responsible for cell cycle arrest of myeloma cells, and downregulation of IL-6 and VEGF levels in the tumor microenvironment, respectively. In addition LD-R may indirectly increase immunotherapy responses in MM patients, stimulated T-cell proliferation and enhancing IL-2, IFγ and TNFα secretion by Th1 cells, as well as downregulating regulatory T cells and upregulating cytotoxic effects of natural killer cells (NKc). We evaluated the numbers of circulating NKc in 15 MM (12 males and 3 females) with a median age of 75 years, during treatment with continuous low dose of LD-R. Seven of these 15 MM patients were newly diagnosed MM patients receiving continuous alternate-day LD-R (10mg) in combination with low dose steroids (15mg), the remaining were MM patients receiving continuous alternate-day LD-R maintenance therapy after autologous stem cell transplantation. Flow cytometry analysis was performed using the Cytomics FC500 (Coulter Miami, FL) to determine the expression of T-cell antigens, NKc antigens. As for as T-cell is concerned, we reported the expression of CD3+CD4+ cells for T-helper, CD3+CD8+ cells for T-suppressor, while for the NKc we recorded the expression of CD3+CD56+ cells and CD3-CD56+ for activated NKc. All antibodies were obtained from Beckman Coulter. Our preliminary data show that prolonged low dose LD-R treatment in MM patients increases circulating NKc further supporting that this drug may mediate its anti-MM effect, at least in part, by modulating NKc number and function.

FINE TUNING OF SURFACE CRLF2 EXPRESSION AND ITS ASSOCIATED SIGNALLING PROFILE IN CHILDHOOD B CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA

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Children with B cell precursor acute lymphoblastic leukemia (BCP-ALL) bearing rearranged cytokine receptor-like factor 2 (CRLF2) may show high rate of relapse. However, CRLF2 rearrangement is associated with its surface over expression and aberrant pSTAT5 and pS6 signalling pathways. Assessment of phenotypic CRLF2 expression and its associated signalling profile may result in a better understanding of the biochemical mechanism underlying such aberrancies. A total of 86 bone marrow samples from children with BCP-ALL were analyzed for CRLF2 expression by flow cytometry (FCM). CRLF2 transcript levels, as well as CRLF2 aberrations (i.e. P2RY8-CRLF2), were analyzed by RQ-PCR. TSLP-induced pSTAT5, pS6, p4EBP1 and pAKT473 were evaluated in leukemia blasts by phosphoflow cytometry. Parallel PCR and FCM measurement of CRLF2 expression was performed: CRLF2+ samples (6/86) by FCM were RQ-PCR overexpressed; similarly all CRLF2- samples (77/86) by FCM were concordantly negative by RQ-PCR. An aberrant TSLP-induced pSTAT5 response was observed in all FCM-CRLF2+ samples tested, and it was significantly different compared to CRLF2 negative cases. Interestingly, we detected a subgroup of CRLF2- patients showing a weak fluorescence shift with an intermediate pSTAT5 response significantly different compared to both the remaining CRLF2 truly negative patients and CRLF2 positive patients. Furthermore we found a TSLP-induced pS6, p4EBP1 and pAKT473 response significantly higher in CRLF2+ patients compared to negative. In conclusion, FCM and RQ-PCR provided high concordance in the identification of patients with CRLF2 over expression. CRLF2+ patients show aberrant pSTAT5 response associated with significant enhanced pS6, p4EBP1 e pAKT473. Interestingly, we identified a subgroup of patients CRLF2- by standard cytometric criteria (i.e <10%), showing low CRLF2 expression detectable by assessing accurate fluorescence shift and significantly higher pSTAT5 response compared to truly negative cases. This findings may open new opportunities in the screening of patients that could benefit of signal transduction inhibitors treatments.

FLOW CYTOMETRY ANALYSIS OF BRONCHOALVEOLAR LAVAGE FLUIDS: EVALUATION OF SCREENING PARAMETERS SUGGESTING THE SUSPECT OF PULMONARY B-CELL NON HODGKIN LYMPHOMA

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To find flow cytometry (FC) screening data on bronchoalveolar lavage fluid (BALF) indicating possible lung B-cell non Hodgkin lymphoma (NHL)(B-NHL), we retrospectively selected BALF with pulmonary B-NHL diagnosis at withdrawal (cases), and at least 6 controls per case, matched for computed tomography. FC screening included total cellularity and CD3-CD4-CD8-CD16/56-CD19 panel, measurements containing 50,000 cell events in the list mode storage. Data detected in cases were compared with the median corresponding values of data detected in controls (Wilcoxon test for paired comparisons).
After a median of 18 months, 6 cases (2 mantle cell NHL, 1 Burkitt-like NHL, 1 B-cell chronic lymphocytic leukemia, 1 diffuse large B-NHL, 1 follicular NHL) were identified (2, ground glass pulmonary infiltrates; 2, enlarged mediastinal lymph nodes plus parenchimal densities; 1, nodules; 1, parenchymal density plus nodules). After a median of 21 months, 56 (median per case: 10) matched controls (41, infection; 4, sarcoidosis; 3, solid tumor; 8, other) were identified. As compared to controls, cases showed significantly higher median lymphocyte (Ly) event number {27,869 [interquartile range (IQR) 22,234-40,055] vs 12,103 (IQR 8,671-12,924)}, median % of Ly on total leucocytes [65% (IQR 62%-69%) vs 26% (IQR 19%-34%)], median B Ly event number [997 (IQR 224-2,814) vs 110 (IQR 49-160)], median % of B Ly on total leucocytes [2.9% (IQR 0.7%-4.7%) vs 0.2% (IQR 0.1%-0.3%)], and median T Ly event number [26,657 (IQR 21,699-36,588) vs 11,530 (IQR 7,250-11,964)](each P, <.05). Total cellularity (319,900/mL vs 232,053/mL), leucocyte (49,134 vs 49,180) and NK Ly (374 vs 270) events, % of T (58% vs 25%) and NK (0.9% vs 0.7%) Ly on total leucocytes, % of T (92% vs 93%), B (4.6% vs 1%), NK (1% vs 2.3%) Ly on total Ly did not differ significantly between cases and controls.
B Ly as event number and as % of leukocytes in BALF seem indicative of performing deepened B-cell phenotying to look for lung B-NHL.

FLOW CYTOMETRY SCREENING OF LYMPHOCYTE SUBPOPULATIONS IN BRONCHOALVEOLAR LAVAGE FLUIDS: EVALUATION OF PARAMETERS INDICATIVE OF IN-DEPTH IMMUNOPHENOTYPE ANALYSIS IN THE SUSPECT OF PULMONARY T-CELL NON HODGKIN LYMPHOMA

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Flow cytometry (FC) screening data on bronchoalveolar lavage fluid (BALF) suggesting in-depth phenotype leading to prompt lung T-cell non Hodgkin lymphoma (NHL)(T-NHL) diagnosis are undetermined.
Among BALF with available follow-up, we selected cases with pulmonary T-NHL and at least 6 controls per case with definite non hematologic disease, matched for computed tomography. FC screening included total cellularity and CD3-CD4-CD8-CD16/56-CD19 panel, measurements containing 50,000 cell events in the list mode storage. FC data detected in cases were compared with the median corresponding values of data detected in controls (Wilcoxon test for paired comparisons).

After a median of 5 months, 6 cases (each, peripheral T-NHL, n.o.s.) were identified (1, enlarged mediastinal lymph nodes; 2, parenchimal densities; 2, ground glass pulmonary infiltrates; 1, parenchimal densities plus ground glass infiltrates). After a median of 17 months, 62 (median per case: 11) matched controls (40, infection; 6, sarcoidosis; 5, drug-induced pneumopathy; 4, bronchiolitis; 2, graft rejection; 1, thromboembolism; 1, lung cancer) were identified. As compared to controls, cases showed significantly higher median lymphocyte (Ly) event number {18,761 [interquartile range (IQR) 18,611-19,392] vs 6,545 (IQR 5,509-10,581)}, median % of Ly on leucocytes [41% (IQR 35%-58%) vs 15% (IQR 10%-23%)], median T Ly events [18,126 (IQR 17,756-18,537) vs 6,162 (IQR 5,099-10,112)], and median % of T Ly on leucocytes [41% (IQR 35%-57%) vs 12.5% (IQR 8%-19.5%)](each P, <.05). Total cellularity (193,736/mL vs 265,405/mL), leucocytes (46,378 vs 48,186), B (33 vs 76) and NK (215 vs 146) Ly events, % of B (0.1% vs 0.1%) and NK (0.2% vs 0.3%) Ly on leucocytes, % of T (96% vs 93%), B (0.2% vs 1%), NK (1.4% vs 3.7%) Ly on total Ly did not differ significantly between cases and controls.
BALF T Ly as events and as % of leukocytes indicate the suspect of lung T-NHL, and the need of further deepened FC analysis.

PROGNOSTIC VALUE OF FLOW CYTOMETRY EVALUATION OF BONE MARROW DYSPLASIA IN LOW-GRADE MYELODYSPLASTIC SYNDROMES

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The current WHO classification of myelodysplastic syndromes (MDS) is based on morphological evaluation of marrow dysplasia. In clinical practice, the reproducibility of the recognition of dysplasia is poor especially in cases with low-grade disease that lack specific markers such as ring sideroblasts and clonal cytogenetic abnormalities. We recently designed a flow cytometry score (FCM-score) applicable in many laboratories that may help to establish the diagnosis of MDS (Haematologica. 2012;97:1209-17). The FCM-score is based on four highly reproducible parameters analyzable from one cell aliquot, i.e. CD34+ myeloblasts (%), CD34+ B-cell progenitors (%), myeloblast CD45 expression, and channel number of side scatter where the maximum number of granulocytes occurs.
In this multicentric study we aimed at evaluating the prognostic effect of FCM-score in a cohort of low-grade MDS. We studied 214 patients affected with refractory cytopenia with unilineage dysplasia (n=58), refractory cytopenia with multilineage dysplasia (n=133), sideroblastic anemia (n=18) and MDS with del5q (n=5). Patients were stratified according to International Prognostic Scoring System (IPSS). FCM-score value was 0 in 44 patients (21%), 1 in 39 patients (18%), 2 in 69 patients (32%), 3 in 46 patients (21%) and 4 in 16 patients (7%). We confirmed a high inter-observer reproducibility of all cytometric parameters included in the score (CV ranging from 11 to 19%). A FCM-score ≥3 was significantly associated with the presence of morphological multilineage dysplasia (p<.001), transfusion-dependency (P<.001) and unfavorable cytogenetics (P=.007), leading to an higher IPSS risk (P<.001). 5- year probability of survival was 74%, 65% and 17% in patients with FCM-score value <2, 2 and ≥3, respectively (P=.003), while 5-year probability of leukemia-free survival was 89%, 78% and 47%, respectively (P=.004). In a multivariable analysis including age, sex and IPSS risk as covariates, FCM-score significantly affected the probability of overall and leukemia-free survival (HR 1.42 and HR 1.56, respectively; P<.001). The likelihood ratios test (P<.001) showed that FCM-score significantly improve the MDS prognostic stratification provided by IPSS. These results indicate that immunophenotyping based on four parameters with high inter-observer reproducibility may have important prognostic implications in low-grade MDS.

NEUTROPHILS ARE IMPAIRED IN MULTIPLE MYELOMA BUT NOT IN MGUS

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Background: In Multiple Myeloma (MM) the immune function is impaired but the role of microenvironment on this dysfunction is unknown.
Aims: Evaluation of immunosuppressive properties of mature neutrophils in MM and MGUS.
Methods: In 60 consecutive newly diagnosed MM, 70 MGUS and 30 healthy subjects we evaluated in neutrophils (N) from peripheral blood the phagocytic activity using a commercially available kit (Phagotest R), and the expression of molecule arginase-1 (Arg-1) and PROK-2. We tested the immunosuppressive properties with functional assay, based on in vitro co-culture of N isolated from patients and T-lymphocytes from healthy subjects.
Results: Despite no differences in the absolute number of neutrophils between MM, MGUS and healthy donors, we found a functional impairment in MM not evident in MGUS patients.
The capability of phagocytosis of MM-N was significantly reduced compared to healthy subjects (p<0.001) and MGUS (p<0.0001), and partially restored after induction chemotherapy.
MM-N exhibited an increased expression of ARG-1 compared to MGUS and healthy controls (25.5 vs 6.2 vs 1 fold changes in gene expression, p=0.003), confirmed by functional assay of enzymatic activity of ARG-1, positively correlated with advanced disease. Similarly, MM-N exhibited an increased expression of PROK-2 compared to MGUS and healthy controls (13.8 vs 1.5 vs 1 fold changes in gene expression, p=0.001).
After PHA-P stimulation, T-lymphocytes isolated from healthy donors missed the expression of activation markers such CD71, CD69, CD25, CD3□ in presence of MM-N for 72 hours, and in a less extensive way in presence of MGUS-N.
Summary / Conclusion: Compared to controls, neutrophils obtained from MM patients and to a lesser extend from MGUS patients, have a reduced phagocytic activity and increased ability to suppress lymphocyte activation. These alterations may contribute to impairment of immune functions that characterizes MM patients.

Immunology

HUMAN AMNIOTIC EPITHELIAL CELLS EXPANDED IN SERUM FREE MEDIUM MAINTAIN THEIR IMMUNOMODULATORY PROPERTIES

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Background: In the past years Amniotic Membrane (AM) has attracted great interest as available source of cells with progenitor/stem cell potential and immunomodulatory properties. Clinical application of cellular therapies requires GMP-compliant culture condition: in this perspective serum free culture systems are recommended. The aim of our study was to investigate if human Amniotic Epithelial Cells (hAECs) cultured in serum-free condition preserve their phenotypic and immunomodulatory features.
Methods: hAECs were isolated from term placenta collected from caesarean sections and cultured in standard serum-rich medium (SRM) and serum-free optimized medium (SFM). Morphological observations and cell proliferation rate were assessed. We used flow cytometry Navios Beckman Coulter to evaluate immnophenotype for stemness and epithelial markers expression. To study in vitro immunomodulatory activity, hAEC were co-cultured on PHA-induced PBMCs in SRM or SFM. The effect of hAECs on PBMCs cell cycle and proliferation has been analyzed through flow cytometer and BrdU incorporation. hAECs immune activity has been evaluated also for tolerogenic molecule expression (i.e. HLA-G).
Results: SFM sustained hAECs growth and stem cell potential without any modification in morphology, cell proliferation rate and stemness. hAECs-mediated suppression of activated PBMC proliferation was maintained in SFM: cell cycle analysis confirmed a marked reduction in percentage of cells in S and G2/M phase as well as in SRM. Furthermore, BrdU incorporation rate of activated PBMC decreased after co-culture with hAECs both with SFM and SRM. Finally, HLA-G expression increased similarly for hAECs cultured in SFM or SRM.
Conclusions: AM holds great promise for the development of cell-based therapies in Regenerative and Reparative Medicine. These data indicate that serum is not essential for hAECs culture: serum-free culture conditions could simplify the transition from laboratory to clinical practice.

HCV COINFECTION INTERFERENCE ON NK CELL PHENOTYPE AND FUNCTION IN VIROLOGICALLY HIV INFECTED SUPPRESSED PATIENTS TREATED WITH MARAVIROC

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Background: Differences of activating NK cell receptors at baseline are associated with the outcome of pegIFNa+RBV treatment of chronic HCV infection (CHI). We here studied the relative contribution of HCV virus on NK cell phenotype and function in virologically HIV suppressed infected patients.
Patients and Methods: NK cell phenotype and function were investigated by cytofluorimetry in 60 HIV infected patients. 36 HIV infected patients were treated with Maraviroc (MRV)-containing ART, while 24 patients were on Maraviroc-free regimen (MRV-). 13/60 patients were HCV coinfected.
In HCV+MRV+ patients, the proportion of $CD56^{bright}CD16^{+/-}$ NK cell subset was increased compared to HCV+MRV- patients (*p<0,05), with comparable proportions of $CD56^{dull}CD16^{+}$ NK cells. $CD56^{-}CD16^{+}$ NK cells were lower in HCV+MRV- patients compared to HCV-MRV- (*p<0,05). NKp46 activating receptor expression was reduced in HCV+MRV+ patients compared to HCV-MRV+ (*p<0,05). Expression of HLADR and CD69 activation markers on NK cells were increased in HCV+ coinfected patients compared to HCV-HIV+ patients (*p<0,05) and in MRV+HCV+ cohort of patients (*p<0,05). NK cell IFNgamma production was analysed by reverse ADCC against P815 cell line in presence of monoclonal antibodies anti-NKp46 and -NKp30. HCV+HIV+ coinfected patients showed reduced IFNgamma production compared to HCV-HIV+ infected patients.
Conclusion: Differences in NK cell molecule expression and function capability are present in HCV coinfected patients compared to HIV monoinfected patients and are not treatment dependent.

EVALUATION OF THE DEGRANULATION PROCESS IN DIFFERENT NK CELL SUBSETS AFTER NKp46 STIMULATION

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Natural killer (NK) cells are large granular cytotoxic lym-

phocytes considered part of the innate immune system. They provide rapid responses to virally infected cells and tumor formation. Target cell recognition by NK cells is a highly dynamic process controlled by the integration of signals from multiple receptors that promotes adhesion, cytokine secretion and/or granule polarization and degranulation or inhibits NK cell functions. Among the different NK activatory receptors, NKp46 (although its tumor ligand is not unraveled) is considered one of the most important ones in tumor recognition, but little is known about its trigger action in the different NK cell subsets. The aim of the present work was to define the synergy of NKp46 stimulation with different co-activatory signals (2B4, DNAM-1, CD2) in the degranulation process of different NK cell subsets. Experiments were performed at different days after mononuclear cell isolation on resting or IL-2-cultured NK cells. In order to mimic the engagement of NK cell receptors with target cell ligands we used beads loaded with combinations of antibodies direct against different NK receptors. To evaluate degranulating NK cells, we have detected the surface expression of CD107a (LAMP-1), a protein expressed on the inner surface of lytic granule membranes, with a specific monoclonal antibody. NK cells were then stained with different antibodies (anti-CD16, - CD56, -KIRs and -NKG2A) to distinguish $CD56^{dim}$ from $CD56^{bright}$ subsets or licensed from unlicensed NK cells. We observed that NKp46 preferentially sinergized with 2B4 stimulation on $CD56^{dim}$ resting NK cell subset. As expected, the percentages of degranulating NK cells were significatively lower on unlicensed than on licensed ones in all stimulations. These differences tended be lost when NK cells were cultured for 2-3 days with or without IL-2.

PERSISTENT INTRAHEPATIC Vγ9Vδ2 T-cells IMPAIRMENT IN THE LIVER OF HCV-INFECTED PERSONS

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Hepatitis C virus (HCV) persistence in the host results from inefficiencies of both innate and adaptive immune responses to eradicate the infection. Among innate immune cells, a functional impairment of peripheral Vγ9Vδ2 T-cells was described during chronic HCV infection; this functional defect can be partially restored by using IFN-α, opening the possibility to boost antiviral innate immune response. Unfortunately, no data are available on intrahepatic Vγ9Vδ2 T-cells that instead, may represent the key actors in the anti-HCV response.
Aim of our work was to analyze intrahepatic Vγ9Vδ2 T-cells in chronic HCV-infected patients (HCV) and, as a control, in healthy donors (HD) subjected to gut resection surgery. Phenotypic and functional features of intrahepatic and peripheral Vγ9Vδ2 T cells were analyzed in both liver tissue and blood samples from 17 chronic HCV patients and in 3 healthy control persons by flow cytometry and ELISA assay.
Irrespective of HCV infection, intrahepatic compartment was characterized by a lower frequency of Vγ9Vδ2 T cells than in the peripheral blood, showing an effector/activated phenotype. In contrast, HCV infection induced an increase of PD-1 expression on both peripheral and intrahepatic Vγ9Vδ2 T-cells, probably due to persistent antigenic stimulation. Finally, liver resident Vγ9Vδ2 T-cells showed a drastic reduction of their ability to produce IFN-α both in HD than in HCV. Nevertheless, intrahepatic Vγ9Vδ2 T-cells from HD can be functionally restored by using IFN-α co-stimulation; in contrast a persistent Vγ9Vδ2 T-cell impairment was observed in the liver of HCV-infected persons. Interestingly, a study aimed to evaluate the mechanisms of this process as well as to find new pathways able to restore Vγ9Vδ2 T-cells intrahepatic function may be mandatory.

CHARACTERIZATION OF IPEX AND 'IPEX-like' SYNDROMES: FOXP3 AND RELATED PATHWAY

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IPEX (Immune dysregulation, polyendocrinopathy, endocrinophathy, X-linked) is an autoimmune disorder characterized by severe early-onset enteropathy, endocrinopathy, dermatitis and autoimmune phenomena. It is caused by the alteration of the T regulatory cells function due to mutations in the *FOXP3* gene. Not all patients with IPEX phenotype show *FOXP3* gene mutations (IPEX-like syndrome) and defects on *CD25* and *STAT5b* have been identified in some of them.
Overall, we collected 93 patients with suspect of IPEX syndrome. Considering patients clinical features, Tregs and FOXP3 expression by flow cytometry, *FOXP3*, *CD25* and/or *STAT5b* genes were sequenced.
FOXP3 mutations were identified in 16.1% of the cases and a variable FOXP3 expression by flow cytometry was observed. Low FOXP3 expression levels, compared with healty subjects, where detected even in patients that don't habour any alteration in the gene sequence. Absent expression of CD25 was detected in two patients with *CD25* mutations, confirming that protein expression analysis can be useful to address the diagnosis in CD25 deficiency. One patient was characterized as a STAT5b deficency, moreover a *STAT1* gain of function mutation characterized by a prolonged phosphorylation of the protein after stimulation was recently identified in one patient.
We confirm that flow cytometry is helpful in the characterization of patient with IPEX and IPEX-like syndromes. In particular we are developing the induced STAT phosphorylation assay that will be a powerful tool both in the differential diagnosis with other primary immunodeficiencies with immune dysregulation where STAT pathway defects might have a pathogenetic role and to explain the pathogenetic role of STAT genetic variation identified by molecular analysis.

FOXP3+ AND CD14+ CELLS, TGF-BETA 1 LEVELS, BACTERIAL TRANSLOCATION INDEXES AND LIVER FIBROSIS SCORE: A CORRELATION STUDY IN HIV+, HCV+, HIV+/HCV+ PATIENTS AND HEALTHY CONTROLS

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HIV infection seems to accelerate the progression of chronic C hepatitis. Regulatory T cell (Tregs) are CD4 + T cells expressing the forkhead-winged-helix transcription factor (Foxp3). Alterations in regulatory T cells (Treg) or in transforming growth factor beta 1 (TGF-beta1) levels, as well of bacterial traslocation markers might be involved in the accelerated course of liver fibrosis seen in HIV/HCV coinfected individuals. A cross-sectional study was conducted on 80 subjects: HIV+ (n = 20), HCV+ (n = 20) and HIV/HCV-co-infected (n = 20) pts, and healthy controls (n = 20), older than 18 yrs. Flow cytometric % of $Foxp3^+$ and $CD14^+$ cells and TGF-beta 1 levels (by ELISA) were analyzed in peripheral blood and correlated with liver fibrosis (measured either by biochemical score, FIB 4, or by elastometry). The correlation among: $Foxp3^+$ and $CD14^+$ cells, CD14 soluble levels, TGF-beta 1, IL17 and bacterial DNA products levels (expression of bacterial translocation) was also analyzed. The % of $Foxp3^+$ cells was significant higher in HIV^+ and HIV^+/HIV^+ pts than in both HCV^+ pts and control group ($p< 0.0005$, Kruskal-Wallis test). There was no statistically significant difference for TGF-beta1 in the four groups. FIB 4 values inversely correlated with TGF-beta1 (Rho correlation coefficient -0.38; $p=0.0155$), as well as with liver stiffness values (Rho correlation coefficient -0.31; $p=0.0498$. $CD14^+$ cells and CD14 serum levels were significantly different between HIV^+ pts vs controls, HIV^+/HCV^+ pts vs controls, HCV^+ pts vs HIV^+/HCV^+ pts ($p< 0.0001$, Kruskal-Wallis test). IL17 levels and bacterial DNA products were significantly different between HCV^+ pts vs the others 3 groups. $Foxp3^+$cells were higher in HIV^+ pts, but they did not influence liver fibrosis staging. TGF-b1 levels inversely correlated with fibrosis suggesting a protective effect. HIV^+/HCV^+ pts showed increased levels of bacterial DNA products, CD14 and IL17 expression as compared with the others groups. The correlation between the translocation index and FIB4 suggest that fibrosis stage may be related to an immunoactivation caused by bacterial translocation.

AIRE POLYMORPHISM, MELANOMA ANTIGEN-SPECIFIC T CELL IMMUNITY, AND SUSCEPTIBILITY TO MELANOMA

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Background AIRE regulates T cell repertoire and is involved in susceptibility to melanoma. In this study, we have tested the role mediated by melanoma antigen (MA)-specific T cell immunity, taking advantage of the structural and functional homology of human AIRE and MAGE with their mouse counterparts.
Methods AIRE and MAGEB2 expression was measured by real time PCR in medullary thymic epithelial cells (mTECs) from two strains of C57BL/6 mice bearing selectively one of the two (T or C) allelic variants of rs1800522 AIRE SNP. The apoptosis extent induced by mTECs in MAGEB2-specific T cells and the susceptibility to *in vivo* melanoma B16F10 cell challenge were compared in the two mouse strains.
Results The C allelic variant, protective in humans against melanoma, induced lower AIRE and MAGEB2 expression in C57BL/6 mouse mTECs than the T allele. mTECs from the strain bearing the AIRE CC genotype induced lower extent of apoptosis in MAGEB2-specific syngeneic T cells than mTECs from the strain bearing the TT genotype ($P<0.05$). Vaccination against MAGEB2 induced higher frequency of MAGEB2-specific CTL and exerted higher protective effect against melanoma development in mice bearing AIRE CC than in those bearing the TT genotype ($P<0.05$).
Conclusion These findings show that alternative allelic variants of one AIRE SNP may differentially shape the MA-specific T cell repertoire potentially influencing susceptibility to melanoma.

FLOW CYTOMETRIC EVALUATION OF HLA-G EXPRESSION IN CORD BLOOD

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Background: human umbilical cord blood (CB) is a useful alternative to bone marrow (BM) and mobilized peripheral blood stem cells (PBSC) to rescue haematopoiesis following myeloablative therapy. We hypothesize that a possible higher expression of HLA-G1 molecules might play a role in better host acceptance showed by cord blood transplantation. On this base, we analysed the cellular subsets expressing HLA-G, a well characterized immunoregulatory molecule able to inhibit innate and adoptive immunity.
Materials and methods: to this aim, a flow cytometric evaluation (Beckman Coulter FC 500) of HLA-G1 membrane expression was performed in 50 CB, 50 peripheral blood (PB), 20 BM and 10 PBSC samples from healthy donors.
Resukts: Total mean value of $HLA\text{-}G1^+$ cells was 0.42% for CB compared to 0.04% for PB, 0.05% for PBSC and 0.06% for BM samples, respectively. More in detail, $CD34^+$ $CD45^+$ $HLA\text{-}G1^+$ cells in CB were 7.8% of total compared to 0.29% in PBSC and 0.05% in BM respectively. The $HLA\text{-}G1^+$ stem cells were negative for the markers of commitment. Finally, differing to what occurs in the peripheral blood in which HLA-G1 is expressed by $CD3^+$ cells, the further aliquots of $HLA\text{-}G1^+$cells detectable in CB, PBSC and BM, were mainly $CD19^+$.
Conclusions: hematopoietic stem cells are endowed with potent immunoregulatory activity. Even in partially HLA-mismatched setting and with a limited cellular dosage CB derived stem cells have been shown ability to engraft. In our observations CB is characterized by a significant higher percentage of $HLA\text{-}G^+$ cells, compared to other stem cells sources. HLA-G could be responsible of a "veto-activity" of $CD34^+$ cells and the higher percentage of $HLA\text{-}G^+$ cells in CB might explain a lower incidence of HLA-mismatch related risk.

INTERFERENCE OF HLA-DR STAINING ON CD124 RECOGNITION IN THE IDENTIFICATION OF MYELOID-DERIVED SUPPRESSOR CELLS

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Myeloid-derived suppressor cells (MDSC) are immature myeloid cells that efficiently suppress T cell functions. In the setting of cancer immunology, MDSC are often defined by interleukin-4 receptor (CD124) expression and low/absent HLA-DR expression by monocytes (MØ). During the immunomonitoring of clinical trials, we observed large differences in the frequency of $CD124^+$ MØ depending on different clones to the HLA-DR molecule used. We show that different monoclonal antibodies (MoAb) that recognize different epitopes of the HLA-DR molecule (clones: L243, B8.12.2, G46-6, WR18, and 423L), variably interfere with the binding of anti-CD124 MoAb. The anti-HLA-DR clones G46-6 and WR18 display higher interferences as compared with clones L243 and B8.12.2, for the binding of anti-CD124 MoAb, and the anti-HLA-DR clone 423L does not interfere at all with the binding. The anti-HLA-DR clone G46-6 inhibited anti-CD124 MoAb binding irrespective of which MoAb was added first. Inhibition was dose-dependent. Activating MØ *in vitro* with phorbol 12-myristate 13-acetate/ionomycin or phytohemagglutinin increased CD124 and HLA-DR expression but did not prevent the interference between the inhibitory clones of HLA-DR molecule with the binding of anti-CD124 MoAb. Anti-CD124 MoAb binding to B cells, that express CD124 and HLA-DR, was inhibited by the various anti-HLA-DR MoAb, indicating that CD124 and HLA-DR molecules are close each other, independent of the cell type. These observations could have implications for both research and clinical studies, in particular when dealing with immunomonitoring of clinical trials.

INNATE IMMUNITY ACTIVATION IN VIROLOGICALLY SUPPRESSED HIV INFECTED MARAVIROC TREATED PATIENTS

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Background: The impact innate immunity status of different antiretroviral therapy (ART) protocols on innate immunity needs to be elucidated.
Methods: 68 virologically suppressed HIV patients in ART ≥ 6 months were subdivided as taking (group G1) or not (group G2) maraviroc (MVC). Circulating monocyte and natural killer (NK) cell cells were studied in correlation with clinical parameters.
Results: Frequency of the inflammatory monocytes (iM) was lower in G1 than in G2 group. Decreased mean fluorescence intensity (MFI) of CD38 and increased of PD-L1 were observed on these cells in the G1 group. The HLA-DR+ NK cell frequency correlated positively with both CD38 and HLA-DR MFI but negatively with PD-L1 MFI on iM in the total patient population Several correlations between clinical and immunological parameters were observed such as, in G1 group, a negative correlation between total cholesterol and the HLA-DR+NK cell percentage and a positive correlation between minimum arterial pressure and HLA-DR MFI on iM.
Conclusions: Frequency and activation of inflammatory subsets of innate immunity correlate with co-morbidity risk factors in undetectable HIV-infected patients and are differently counteracted by cART protocols including or not MVC.

BEGEDINA®, CD26 AND GvHD

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Background. Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for the majority of hematological malignancies. Over the years transplant related mortality (TRM) was significantly reduced due to better supportive care, improving HLA-typing and treatment of infections. However, mortality due to steroids refractory acute graft versus host disease (aGvHD) remains high and unchanged. The aim of this study is to evaluate the efficacy of BEGEDINA® in 12 patients with steroids refractory aGvHD grade III-IV after allogeneic HSCT in a pilot study (EUDRACT) and to test the CD26+ expression on circulating CD3+ cells.
Patients and methods.BEGEDINA® is a murine monoclonal antibody against CD26 or Dipeptidyl peptidase IV(DPPIV), an ubiquitous type II transmembrane glycoprotein expressed particularly on CD4+CD45RO+ memory T cells, with binding activities.BEGEDINA® was administered at the dosage of 2 mg/day for 5 consecutive days. Ten patients was grafted from unrelated donor, 2 from HLA-ID sibling; the diagnosis was AL (7), MDS (2), NHL (2) and SAA (1). The expression of BEGEDINA®-CD26 on CD3+ cells was analyzed by flow cytometric evaluation (Beckman Coulter NAVIOS/FC500) on peripheral blood samples, before and after each administration of BEGEDINA®.
Results.Ten patients (91%) showed a very good response to the treatment with BEGEDINA®, 1PR and 1NR. Despite the clinical results, the biological effect is not so clear and the expression of BEGEDINA® CD26 on CD3+ cells was variable (25-90%) between patients and independent from the clinical response.
Conclusions.BEGEDINA® the murine monoclonal antibody against CD26 in vivo is safety and efficacy for the treatment of steroid refractory aGvHD. The expression of CD26 on CD3+ cells is variable and is not related to the clinical response.

COMPARATIVE ANALYSIS OF CANCER VACCINE SETTINGS FOR THE SELECTION OF AN EFFECTIVE PROTOCOL IN MICE

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Cancer vaccineshave not yet reached satisfactory clinical results, probably due to the difficulty to select an efficient tumor associated antigen and immunization protocol. To this issue, we performed a comparative analysis of different gp100 vaccination strategies to obtain protection against experimental melanoma. C57BL/6J mice were challenged subcutaneously with B16F10 melanoma cells, after vaccination with mouse or human $gp100_{25-33}$ peptide plus CpG adjuvant; mouse or human gp100 gene; mouse or human $gp100_{25-33}$ peptide-pulsed dendritic cells (DC). Alternatively, a neutralizing anti-IL10 monoclonal antibody (mAb) was subcutaneously administered at the site of tumor challenge to counteract regulatory cells. Finally, human $gp100_{25-33}$ peptide-pulsed DC vaccination was performed associating with administration of the anti-IL10 mAb. **V**accination with human $gp100_{25-33}$ peptide-pulsed DC was the most effective immunization protocol, although not achieving a full protection. Administration of the anti-IL10 mAb showed also a remarkable protective effect, replicated also in mice challenged with Anaplastic Large Cell Lymphoma. Combining $gp100_{25-33}$ peptide-pulsed DC immunization with IL10 counteraction, a 100% protective effect was achieved. The analysis on tumor infiltrates T cells showed increase of CD4+granzyme+ T cells and decreased number of CD4+CD25+Foxp3+ Treg elements from mice treated with either $gp100_{25-33}$ peptide-pulsed DC vaccination or anti-IL10 mAb. These data suggest that processes of intratumoral re-balance between effector and regulatory T cell subpopulations may play a critical protective role in immunotherapy protocols.

ASSESSMENT OF DOPAMINERGIC RECEPTORS ON HUMAN CD4+ T CELL SUBSETS BY MEANS OF FIVE-COLOR FLOW CYTOMETRY ANALYSIS IN WHOLE BLOOD

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Dopamine (DA) is a key neurotransmitters which acts on DA receptors (DR) classified, according to their pharmacology and biochemistry, into D1-like (DRD1 and DRD5) and D2-like (DRD2, DRD3 and DRD4). Emerging evidence indicates that DA also exerts direct effects on the immune system which are relevant in health and disease. Exploitation of the immune effects of DA requires however better understanding of the pattern of expression of DR on immune cells.
We developed a flow cytometric assay to examine the DR expression on human CD4+ T lymphocyte subsets by use of whole blood. Samples of venous blood were obtained from healthy donors (F/M: 17/8; age [mean±SD]: 55.9±15.7 years). Immunophenotyping of DR on CD4+ T cells was performed by a 5-color flow cytometric analysis in whole blood by use of a two-step protocol: during the first step, cells are stained for DR by an indirect labelling procedure (primary antibody (Ab) + secondary Ab, PE); during the second step, cells were incubated with a cocktail of anti-human CD3, CD4, CD45RA and CCR7 ab conjugated with fluorochromes to identify the subsets of interest. Expression of CD45RA and CCR7 allowed identifying the following subsets: T central memory (Tcm, CD3+CD4+CD45RA-CCR7+), T naive (CD3+CD4+CD45RA+CCR7+), and T effector memory (Tem, CD3+CD4+CD45RA-CCR7-).
CD4+ T cells were (mean±SEM) 66.3±2.4% of total CD3+ T cells. Among CD4+ T cells, Tcm were 28.7±1.9%, Tem 24.8±1.6% and T naive 43.4±2.7%. All five DR were expressed on CD4+ T cells. The D2-like DRD2, DRD3 and DRD4 were expressed by 3.1±0.5%, 6.6±1.1% and 8.1±1.6%,, while the D1-like DRD1 and DRD5 were expressed by 11.7±1.4% and 13.2±1.2% of the cells ($P<0.05$ and $P<0.0001$ vs DRD2, DRD3 and DRD4). The same DR expression pattern was observed in CD4+ T cell subsets, although statistical significance of the differences between D2-like and D1-like DR was reached only in T naive cells ($P<0.05$ for DRD1 and $P<0.0001$ for DRD5).
In conclusion, we developed a flow cytometric assay which allows the straightforward analysis of DR on human CD4+ T cell subsets. Knowledge of DR expression pattern on specific subsets will provide a key contribution to plan experiments aimed at the understanding of the functional relevance of DR pathways in the regulation of the immune response as well as to the interpretation of results. *This study was supported by a grant from Fondazione CARIPLO (Project 2011-0504: Dopaminergic modulation of CD4+ T lymphocytes: relevance for neurodegeneration and neuroprotection in Parkinson's disease - The dopaminergic neuro-immune connection) to MC.*

ANALYSIS OF CTLA-4 EXPRESSION ON PRIMARY MELANOMA CELL LINES AND THEIR REACTIVITY WITH IPILIMUMAB BY FLOW CYTOMETRY

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CTLA-4 (Cytotoxic T lymphocyte antigen-4) is a negative regulator of T cell activation. The blocking of CTLA-4 with monoclonal antibodies, such as Ipilimumab, is currently used in the treatment of metastatic melanoma to increase antitumor immunity. Herein, we have analyzed CTLA-4 expression and Ipilimumab reactivity on 17 melanoma cell lines (14 primary and 3 long-term cell lines) by flow cytometry. Expression of surface CTLA-4 was analyzed by direct immunofluorescence using a PE-conjugated goat anti-CTLA-4 polyclonal antiserum (R&D Systems), whereas cytoplasmic CTLA-4 was analyzed by indirect immunofluorescence using the unconjugated anti-CTLA-4 murine mAb (14D3 clone, from eBioscience). Next, we tested the reactivity of the therapeutic human mAb Ipilimumab (Bristol-Myers-Squibb) both on the surface and in the cytoplasm of the melanoma cell lines followed by the addition of Alexafluor 647-conjugated goat anti-human IgG as secondary antibody (Molecular Probes, Inc.). Appropriate negative controls were included. The flow cytometric analyses were performed with a FACSCalibur cytometer (Becton Dickinson) using CellQuest software (Becton Dickinson). We found that all melanoma cell lines, except FO-1, expressed variable levels of surface and cytoplasmic CTLA-4 with both commercial and Ipilimumab antibodies. CTLA-4 expression was confirmed at trascriptional level by RT-PCR and by immunohistochemistry on 33 melanoma tissues. We also

observed that Ipilimumab can trigger innate immunity in terms of antibody dependent cellular cytotoxicity (ADCC) or Tumor Necrosis Factor (TNF)-α release. Finally, a xenograft murine model was set up to determine *in vivo* the effects of Ipilimumab and NK cells on melanoma. Our studies demonstrate that CTLA-4 is expressed not only on T cells but also on tumor cells and Ipilimumab triggers effector lymphocytes to cytotoxicity and TNF-α release. These findings suggest that Ipilimumab can directly activate the elimination of CTLA-4$^+$ melanomas.

NUMERICAL ALTERATIONS OF DENDRITIC CELL SUBSETS IN THE PERIPHERAL CIRCULATION OF PATIENTS WITH PARTIAL 22q11.2 DELETION SYNDROME

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The immune deficiency of the 22q11.2 deletion syndrome (formerly called DiGeorge or velocardiofacial syndrome (DS)), is due to impaired development of the thymus gland; therefore, the majority of the studies have focused on T cell numbers and function. Furthermore, a subgroup of patients with partial (p)DS appears to be at higher risk for relevant infectious and autoimmune complications.
The number and function of dendritic cells (DCs) in the 22q11.2 DS has not been investigated, although these cells represent the most important family of professional antigen-presenting cells, able to efficiently prime naïve, memory and effector T cells and are responsible for activating also natural killer T (NKT) cells.
Therefore, we have enumerated circulating DCs in 13 patients with partial 22q11.2 DS, directly from whole blood using a four-colours flow cytometric method. DCs are also examined for the expression of CD85k antigen because it is down-regulated following DC activation and up-regulated in tolerogenic DCs, leading to the induction of T reg cells.
We report a significant decrease in the absolute number of total DCs compared to age-matched healthy controls, which affects both myeloid and plasmacytoid DC (mDC and pDC) subsets.
In particular, 9 out of 13 patients have a total DC number under -1SD of control mean. Interestingly, the patient with the lowest mDC value suffered from metastatic thyroid cancer. The mean fluorescence intensity (MFI) of CD85k on mDCs is significantly higher in patients compared to controls, reaching in 7 patients values higher than +2SD of the mean of the controls.
We suggest that the reduction of DCs could contribute to the immunodeficiency of this syndrome; additional investigations are necessary to dissect the heterogeneity observed in this population in order to early detect the subgroup of patients at higher risk of infections, tumours and/or autoimmune disorders.

DISTINCTIVE FEATURES OF CLASSIC AND NON-CLASSIC (Th17 derived) HUMAN TH1 CELLS

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T helper17 (Th17) lymphocytes represent a third arm of the CD4+ T-cell effector responses, in addition to Th1 and Th2 cells. Th17 cells have been found to exhibit high plasticity because they rapidly shift into the Th1 phenotype in inflammatory sites. In humans, Th1 cells derived from Th17 cells express CD161, whereas classic Th1 cells do not; these Th17-derived Th1 cells have been termed nonclassic Th1 cells. In this study, we examined similarities and differences between classic and nonclassic human Th1 cells by assessing a panel of T-cell clones, as well as CD161+ or CD161– CD4+ T cells derived ex-vivo from the circulation of healthy subjects or the synovial fluid of patients with juvenile idiopathic arthritis. The results show that nonclassic Th1 cells can be identified based on CD161 expression, as well as the consistent expression of retinoic acid orphan receptor C, IL-17 receptor E, CCR6, and IL-4-induced gene 1, which are all virtually absent in classic Th1 cells. The possibility to distinguish these two-cell subsets by using such a panel of markers may allow the opportunity to better establish the respective pathogenic roles of classic and nonclassic (Th17 derived) Th1 cells in different chronic inflammatory disorders.

THE IMMUNOSUPPRESSIVE NETWORK: CIRCULATING MYELOID-DERIVED SUPPRESSOR AND T REGULATORY CELLS IN PATIENTS WITH NEOADJUVANT SHORT RT RECTAL CANCER PATIENTS

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Myeloid-derived suppressor cells (MDSCs) are a heterogeneous family of myeloid cells that along T regulatory cells (Treg) play a major role in antitumor immune response of cancer patients. In colorectal cancer patients, MDSCs have recently been described as correlating with stage, metastasis and chemotherapy response. A therapeutic option for locally advanced rectal cancer (LARC) is neoadjuvant treatment based on short course radiotherapy based on 5 fractions of 5 Gy for 5 days (NRT). Since MDSCs have been characterized as a population of cells that can regulate Treg cells, the overlapping target cell population of Treg cells and MDSCs is indicative for the importance and flexibility of immune suppression under pathological conditions. Aim of this work was to evaluate the value of MDSCs and Treg cells in peripheral blood LARC subjected to NRT and then surgery by Multicolor Flow Cytometric Analysis, to identify possible pattern of immune modulation. Thirteen patients were evaluated for both circulating MDSCs and Treg cells *s-I*) at time 0, *s-II*) after two weeks from the beginning of RT), *s-III*) after 5 weeks from the beginning of radiotherapy; s-*IV*) before surgery and s-*V*) 24 hours post surgery and s-*VI*) 6 months after surgery s-*VII*) 12 months after surgery. The analysis of MDSC cells (identified as granulocytic LIN$^-$/HLA-DR$^-$/CD33$^+$/CD15$^+$/CD11b$^+$ and monocytic CD14$^+$/HLA-DR^{low-}/CD11b$^+$/CD33$^+$) showed a reduction in all of 13 patients due to NRT, with a minimum level at pre-surgery sample (*s-IV*), more markedly in poor-responders 6/13 (TRG III and IV) compared to responders patients 7/13 (TRG I and II), this difference was statistically significant ($p = 0,037$ repeated measure Anova Test); the number of MDSC cells showed a recovery to baseline levels at 12 months after surgery (s-*VII*) for all patients. The value of Treg cells CD4$^+$/CD25^{hi+}/ FoxP3$^+$ showed (although not significantly) a progressive strong increase in poor-

responder patients compared to the decrease in responders patients, with the respective maximum and minimum peaks to the pre-operative evaluation (*s-V*); after 12 months after surgery (s-*VII*) these levels remain substantially different. Thus a clear immune response is detectable in LARC according to NRT, it seems that MDSC value could represent an early marker of response to RT; moreover, an opposite trend between MDSC and Treg could be a useful indicator of poor prognosis.

FINGOLIMOD MODULATES PERIPHERAL EFFECTOR AND REGULATORY T CELLS IN MS PATIENTS

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Multiple sclerosis (MS) is a complex neurological disease where, in genetically predisposed individuals, the unbalanced interplay between pathogenic and regulatory T cells will result in the progression of the autoimmune assault to neural antigens. Fingolimod (FTY720), an oral sphingosine 1-phosphate modulator recently approved for the treatment of MS, inhibits the egress of T cells from lymph nodes acting specifically on naïve and memory T cells and sparing effector T cells. Here we characterized IL-17 and IFN producing effector CD4 and CD8 positive T cells as well as CD4 positive $CD25^{high}CD127^{low}$ regulatory T cells in MS patients before and one month after treatment was started. We observed that did not significantly affect the percentage of CCR6 and CD161 positive T cells in both CD4 and CD8 subsets, while it significantly reduced the levels of both CD4+CCR6+CD161+ and CD8+CCR6+CD161+ producing IFN alone or in combination with IL-17. The IL-17 secreting cell percentage in both subsets was affected by the treatment to a lesser extent. Finally, we observed that $CD4+CD25^{high}CD127^{low}$ regulatory T cells were decreased in MS patients compared to healthy controls and significantly increased their frequencies. These findings demonstrate that FTY720 functionally modulates the production relevant pro-inflammatory cytokines in potentially pathogenic effector cells and increases the number of circulating regulatory T cells possibly contributing in restoring a balance between these populations.

KIR2DS1-DEPENDENT ACQUISITION OF CCR7 AND MIGRATORY PROPERTIES BY HUMAN NK CELLS INTERACTING WITH ALLOGENEIC HLA-C2+ DC OR T CELL BLASTS

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Natural killer (NK) cells may capture the CCR7 chemokine receptor from allogeneic $CCR7^+$ cells by a mechanism termed trogocytosis and acquire migrating properties in response to the CCL19/CCL21 lymph node chemokines. This event is negatively regulated by inhibitory KIRs and NKG2A. In this study, we analyzed the role of activating KIRs in the process of CCR7 uptake in NK cells interacting with different types of allogeneic $CCR7^+$ cells. Analysis of freshly isolated peripheral blood NK cells and NK cell clones revealed that in $KIR2DS1^+$ alloreactive NK cells, co-cultured with $HLA\text{-}C2^+$ $CCR7^+$ lymphoblastoid cell lines, the interaction of KIR2DS1 with HLA-C2 (its ligand) resulted in increased CCR7 uptake. We show that KIR2DS1 represents a remarkable advantage for the acquisition of CCR7 also from $HLA\text{-}C2^+$ allogeneic DC and T cell blasts because of its ability to override inhibition by NKG2A. These findings have important implications in haploidentical haematopoietic stem cell transplantation in which donor-derived (alloreactive) $KIR2DS1^+$ NK cells upon acquisition of CCR7 become able to migrate towards lymph nodes, where they may kill patient DCs and T cells, preventing graft-versus-host and host-versus-graft reactions.

NEW IMMUNOASSAY TO MEASURE INTESTINAL AUTO-ANTIBODIES

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In coeliac disease (CD) anti-tissue transglutaminase autoantibodies (anti-tTG) were synthesized in the intestinal mucosa, even when absent in serum, and can be used in the diagnosis of CD especially for those seronegative subjects with high risk for CD.

The aim of our study was to develop a new operator-independent assay to measure intestinal anti-tTG in comparison with the double coloured immunofluorescence staining in detecting the IgA anti-tTG deposits on the intestinal biopsies.

We enrolled 153 consecutive patients who underwent upper gastrointestinal endoscopy at Children's Hospital. Two biopsies were obtained: one for double coloured immunofluorescence staining and one for acid antibody extraction. The eluted samples were buffered and quantified for total protein concentration. 50μg of proteins from each sample were incubated with magnetic beads coated with human tissue transglutaminase to capture anti-tTG antibodies. Beads were incubated with anti-human IgA FITC and analyzed by flow cytometry. The flow cytometry cut off value was calculated as the media ± one standard deviation of the percentage of fluorescent beads obtained from CD negative intestinal samples.

We calculated the cut off value of 15%. We found 84 positive samples (mean 77±22 s.d.) and 69 negative results (mean 2,3 ±3,4 s.d.). In double coloured immunofluorescent assay we had 85 positive results and 68 negative ones. In comparison with the latter assay the beads assay had a sensitivity of 95% and a specificity of 95%. Among the study population there were 5 asymptomatic subjects with no serological markers, without any intestinal damage who were both positive for IgA anti-tTG intestinal deposits in double coloration fluorescence assay and beads assay. These patients were put to gluten free diet.

Our preliminary results suggest that beads assay is an alternative operator-independent assay to measure anti-tTG deposited in the intestinal mucosa also in the very early stage of CD. This assay can also be adapted to detect other autoantibodies in any tissue samples.

EVALUATION OF LYMPHOCYTE ACTIVATION MARKERS, POST-THYMIC NAIVE AND MEMORY T CELLS SUBSETS AND HLA LOCI B,Cw FOR THE MONITORING OF HIV INFECTION IN THE ACUTE PHASE

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Many authors agree on the need, to identify additional cytofluorimetrics markers beyond CD4+ and CD8+ subsets of T cells, for assessing lymphocyte activation and evaluate post-thymic naïve and memory cell subsets expression, in naïve HIV patients undergoing current treatments. It has been previously shown the ability of alleles B57-58, B27 and Bw4 to mediate a CD8+ cytotoxic control mechanism in HIV-positive patients, using CD38, HLA-DR and CD45RO. In this study, we evaluated activated CD4+ and CD8+/HLA-DR+/CD38+ cells, post-thymic naïve CD4+ and CD8+/CD45RA+/CD62L+ cells and memory CD4+ and CD8+/CD45RA+/CD62L- cells on naïve HIV-positive patients both before and after starting HAART therapy.12 HIV+ blood samples from Infectious Diseases ward and 12 blood samples from donors at Terni Blood Bank were studied using FacsCANTOII BD at T0 and T1, T2 and T3 post HAART treatment. They were incubated with TBNK (BD), CD8FITC, CD38PE, CD3PerCP, HLA-DR APC as activation markers, with CD45RA-FITC, CD62L-PE, CD3PerCP, CD8APC for naïve and memory cells, and analysed using the Canto and DIVA software programs. For serological typing of HLA, we used Biotest plates and Dynal Magnetic Beads. 5 HIV naïve patients showed high levels of CD8/CD38/HLA-DR and of naïve and memory cells. In those patients a decrease in HIV-RNA was achieved wich did not correlate with a decrease CD8/CD38/HLA-DR and naïve and memory cells levels. This was probably due, as stated in the literature, to HBV, HCV and TBC coinfection, confirming persistent T cells activation. The remaining 7 HIV+ patients, showed a reduction in CD38/CD8 levels of expression and CD4 and CD8 memory cells, as expected after HAART terapy, with similar trends at 4-8-12 months post treatment (stastically significant p=0,025 of Student Tests). Patient 5 with haplotype B35 and CW4 showed a depletion of CD4 and CD8, of naive post-tymyc and memory T cells subsets and activation markers. Patients with alleles B57 and Bw4 showed a response to therapy with lowering of viremia and a persistent cytotoxic activation mechanism.

COMBINATION THERAPY WITH IPILIMUMAB AND ELECTROCHEMOTHERAPY (ECT): PRELIMINARY EFFICACY RESULTS AND CORRELATION WITH T REGULATORY CELLS (Treg)

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Background: Ipilimumab is the first agent approved for the treatment of advanced melanoma that showed a survival benefit in randomized phase 3 trials. It is associated with a slow onset and low rate of responses and, in many cases, responses occur after other therapies, like targeted therapy and radiotherapy. ECT has been shown to be effective for treatment of cutaneous and subcutaneous melanoma metastases. The current challenge is to improve Ipilimumab efficacy in combination/sequence with ECT to verify the possible increase of response rate. Furthermore, we evaluated the T-Reg variations in peripheral blood mononuclear cells (PBMC) of treated patients.
Methods: We analyzed data from 10 patients (pts) with advanced melanoma (6 stage IIIc and 4 stage IV M1c). They were treated with ipilimumab at 3 mg/kg every 3 weeks for 4 cycles (day 1) and ECT with bleomycine at 15 mg/m2 (day 2) on superficial lesions. PBMCs were collected on day 0, 1 and 2, then on day 15 and 30 from ECT, at each cycle of ipilimumab and at every tumor evaluation (every 12 weeks). T-Reg were analyzed by FACS analisys labeling PBMCs with anti-CD4, CD25 and anti-FoxP3.
Results: 10/10 (100%) pts showed local objective responses (4 CR and 6 PR). 7/10 (70%) pts showed local response (6 PR and 1 CR) after the second ipilimumab dose and 3/10 showed response (3 CR) at week 12. Two pts with PR (28%) out of the group of 7 showed response on distant lesions at week 24 (abscopal effect). To date all pts are still alive with a median follow up of 11 months (range 6-18). In all pts a decrease of T-Reg of 0.10% (range 0.50-2.6%) per cycle and no variation of CD4+ and CD25+ lymphocytes has been observed.
Conclusion: The preliminary results show that the combination approach with Ipilimumab and ECT could improve the responses compared to the Ipilimumab treatment alone. The decrease of circulating T-Reg cells could be associated with better response to treatment. Further studies about this combination are warranted.

CORRELATION BETWEEN FLC AND CYTOMETRIC EVALUATION OF PLASMACELLS IN MONOCLONAL GAMMAPATHY AND IN PATIENTS WITH MULTIPLE MYELOMA: A CLINICAL CASE EXEMPLARY

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BACKGROUND. Flow cytometry and immunological methods have allowed the characterization of a series of surface antigenic molecules expressed on either MM or normal cells. With this techniques several molecules differentially expressed on normal and MM cells and correlated with the prognosis of MM patients have been identified. Normally, the light chains are produced in excess and therefore a fraction is present in the serum in free form (FLCs). In monoclonal gammapathy, the production of FLC type k o λ exceeds the normal concentrations also altering the ratio κ / λ (rFLC).
MATERIALS AND METHODS.We have studied a series of 60 patients with monoclonal gammopathy(40 MGUS and 20 MM)and followed for about 6 months with periodic evaluations of ESP, IFE, FLC assays and bone marrow aspirate. Among our data there is also a difference (dFLC) between free light chains involved in the pathology (iFLC) and uninvolved free light chains (uFLC), which is important in monitoring patients in therapy.The

immunophenotype of bone marrow plasmacells demonstrated that 10/40 MGUS were CD56 positive while 20/20 MM were CD56 positive. We have extrapolated the case specimen of a seventy patient whose MM was diagnosed in November 2011.
RESULTS AND CONCLUSIONS. In the patient considered we have observed an excellent therapeutic response through the densitometric evaluation of the CM in association with the assay of serum FLC, which correlates directly with the number of bone marrow plasma cells. In correspondence of the increases in dFLC bone marrow plasmacells increase in number and increase the positivity of CD56 on their surface.
As expected, the plasmacell movement is directly related to the increase in dFLC. This assay allows however to monitor in a reliable, sensitive and certainly less invasive way than bone marrow aspirate the trend of the disease. This assay allows to reduce the number of IFE and bone marrow aspirates reserving these investigations to the occasions in which the dFLC increases.

LONG-TERM WHOLE BLOOD DONATION CAN AFFECT THE BALANCE BETWEEN NAIVE AND MEMORY T LYMPHOCYTE POPULATIONS IN HUMAN PERIPHERAL BLOOD

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To provide protection against both unknown new pathogens and recurrence of previous infections, the immune system maintains adequate populations of naive and memory T cells, respectively. However, thymic output of naïve T cells is drastically reduced in human adults. We hypothesized that long-term frequent donation of whole blood could constitute a model of chronic T cell depletion. Thereby, we asked whether such cell loss may have an impact on the naive T cell reservoir and peripheral T cell subsets homeostasis. To evaluate the composition of circulating T lymphocyte in relationship to high numbers of donations, 55 donors with a median age of 58 who had donated >85 times were enrolled in this study along with 55 age-matched donors who had donated <15 times. The study has been performed by flow cytometry on samples of red-cell lysed whole blood labeled by staining with CD3, CD8, CD28, and CD95 antibodies. Stained samples have been acquired through a FACSCalibur flow cytometer and analyzed by CellQuest Software. Percentages of naïve T cells in peripheral blood of long-term donors were significantly lower than in donors who donated few times. In particular, in long-term donors, naive (CD95-CD28+) T cells represented 22% (± 7.4 SD) of gated CD3+ T cells, whereas in donors who donated few times they represented 36% (± 11.2 SD) ($p<0.001$, Mann-Whitney test). Conversely, effector memory (CD95+CD28-) T cells were significantly more abundant in long-term donors (27% ± 12.5) as compared to occasional donors (15% ± 9.7) ($p<0.001$). By contrast, central memory T cells (CD28+CD95+) did not differ between the two groups. Differences between the two donors' groups were more pronounced within the CD8+ than the CD4+ T cell subset. Results from this preliminary study suggest that an high number of donations of whole blood can reduce the proportion of the naïve T cell reservoir in peripheral blood while homeostasis of T cells is maintained through expansion of effector memory T cells.

Methodology and Technology

BIOCOMPATIBILITY ANALYSIS OF NOVEL (butylene 1,4-cyclohexanedicarboxylate) BASED ELECTROSPUN COPOLYESTERS CONTAINING PEG-like SEQUENCES FOR TISSUE ENGINEERING APPLICATIONS

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Tissue engineering aims to regenerate damaged tissues, instead of replacing them, by developing biological substitutes that restore, maintain or improve tissue function. It relies extensively on the use of porous 3D-scaffolds to provide the appropriate environment for the regeneration of tissues and organs. The scaffolds, which essentially act as a template for tissue formation, were produced from a variety of biomaterials and manufactured using a plethora of fabrication techniques. Among these last, electrospinning is the only method capable of producing continuous polymer nanofibres. Electrospinning is a unique technology that can produce non-woven fibrous articles with fiber diameters ranging from tens of nanometers to microns. We propose a new class of aliphatic polyesters based on poly(butylene 1,4-cyclohexandicarboxylate) (PBCE). In particular, random copolymers containing PEG-glycol subunits have been prepared, fully characterized and subjected to electrospinning in order obtain fibrous scaffolds to be used in tissue engineering applications. In this study, we aimed at investigating skeletal myogenesis on these novel electrospun scaffolds. The potential effects of all tested copolymers were evaluated by investigating C2C12 proliferation (via CCK8 assay) and differentiation (via MHC immunostaining). We demonstrated that they enable skeletal myogenesis *in vitro* by aiding in (a) myoblast adhesion and proliferation, and (b) myotube alignment, by providing the necessary directional cues along with architectural and mechanical support. Further studies will be conduct in order to demonstrate their application in the tissue engineering field.

***IN VITRO* COMPARATIVE STUDY OF THE EFFECTS OF ZINC-CONTAINING BIOACTIVE GLASS-COATED TITANIUM ON PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS**

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Bioactive glasses coating of titanium (Ti) scaffolds are very attractive materials for bone regeneration due to their versatile properties. An important feature of bioglass it that, upon implantation, its surface undergoes complex kinetic modifications leading to the formation of an amorphous calcium phosphate layer. Bioactive glasses can be improved with trace quantities of elements such as Cu, Zn and Sr, which are known to be beneficial for healthy bone growth. The aim of this study was to *in vitro* evaluate the biological reactivity of bioglass coating titanium containing 0.4% wt ZnO in terms of hMSC proliferation and differentiation. The mesenchymal stem cells isolated from adult human bone marrow (hBMSC) or from human adipose tissue (hASC) was seeded on scaffolds. MTT assay was performed to assess the biocompatibility of the scaffolds while the distribution of the cells inside the constructs was analyzed by Scanning Electron Microscopy (SEM). The osteo-inductive potential of these scaffolds was investigate by the immunolocalization of osteogenic markers (i.e. Alkaline phosphatase, ALP) using Confocal Laser Scanning microscopy (CLSM). In comparison with uncoating titanium and bioglass coated 3D Ti 58S, the 58S-Zn0.4 bioglass-coated 3D Ti scaffold showed a significant increase in cellular proliferation. SEM analysis showed that cells more homogenously covered the surface of 58S-Zn0.4 disk than to 58S and uncoating titanium disks. As shown by CLSM, ALP production was higher with respect to titanium and 58S. We conclude that the treatment of titanium surface with bioglass and 0.4%wt zinc oxide, improves its ability to enhances mesenchymal stem cells adhesion, proliferation and their osteogenic differentiation.

COMPARISON BETWEEN WESTERN BLOT AND FLOW CYTOMETRY IN THE DETECTION OF PTEN AND mTOR IN PEDIATRIC T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Signalling networks such as the PI3K-AKT-mTOR pathway play a role in the modulation of the aggressiveness of T-Cell Acute Lymphoblastic Leukemia (T-ALL), but the prognostic significance of an aberrant activation of this pathway is poorly investigated. Western Blot (WB) represents the method of references to study proteomic profile in hematological malignancies, but Flow Cytometry (FC) can also be applicable at single-cell level in the same setting (Irish JM *et al*, Nat Rev Cancer 2006), however the choice of the best method can be still an open question. We analyzed the PTEN and mTOR basal expression in bone marrow (BM) samples from 9 children with T-ALL and 9 normal subjects1}. Western blot analysis was performed using standard RIPA buffer for protein extraction. A StripAblot Stripping Buffer was used to recover membranes. Ratios between target protein over beta-actin have been calculated by densitometry analyses, using Alliance instrument and Uviband software. Samples were examined also by FC as previously described (Gaipa G *et al*, Leukemia 2008), and protein levels were measured as % of positive cells compared to isotype control. Positivity or negativity by WB was established by presence or absence of protein band, while for FC threshold for positivity was set at ≥ 1% of positive cells. Qualitative concordance (positive *Vs* negative) was 100% using the two methods, being 3/ 9 and 2/9 T-ALL samples PTEN and mTOR negative, respectively. Mean values of PTEN/mTOR ratio in T-ALLs was highly concordant when measured by FC or WB: 5.40 and 5.57 respectively, and this result was highly reproducible when individual paired samples (n=4) were considered. Nine normal BM samples were also analyzed by FC: 7/9 and 9/9 were PTEN and mTOR-positive, respectyively. Interestingly PTEN was significantly higher in T-ALLs as compared to normal BM: 47.00%±26.81% *Vs* 4.30%±2.89% (p=0.001), by contrast mTOR was higher in normal BM as compared to T-ALLs: 8.70%±3.54% *Vs* 29.70%±30.22% (p=0.06), consequently PTEN/mTOR ratio resulted reversed in the two subgroups: 5.40 in T-ALLs and 0.14 in normal BM samples. Moreover, when only CD7+ T lymphocytes (instead of total nucleated cells) were analyzed in normal BMs 9/9 were PTEN negative by FC (<1,0%), while in T-ALL CD7+/CD45 low blasts resulted either PTEN negative (3/9) or over expressed (6/9, mean PTEN positive cells: 47.00%, range 18.4%-90.5%). Our results showed that WB and FC techniques provide full concordant qualitative (positive *Vs* negative) read outs in the measurement of both PTEN and mTOR in T-ALLs, and highly concordant PTEN/mTOR ratio measurements. Of note, PTEN/mTOR ratio in normal BM and in T-ALLs are inversely correlated and this observation was not previously reported, suggesting further investigations.

EVALUATION OF DIELECTROPHORESIS FOR ISOLATION OF PANCREATIC ISLETS FROM EXOCRINE TISSUE

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Dielectrophoresis (DEP) has been reported as a promising method for cell manipulation without physical contact in miniaturized lab-on-chip devices, since it exploits the dielectric properties of cells suspended in a fluid and subjected to high-gradient electric fields. Objective of this work is to demonstrate the applicability of DEP for the purification of Langerhans islets from fragments of pancreatic exocrine tissue, a critical stage for the further transplantation of insulin secreting islets in patients affected by type I diabetes. A critical aspect of the application of this physical phenomenon to the manipulation of pancreatic islets is related to their size. DEP is usually applied for the manipulation of single cells, with dimensions of the order of microns, while Langerhans islets are aggregates of cells and have a minimum diameter of 100 µm. Dielectric models of single pancreatic beta cells and Langerhans islets as cell aggregates were developed. Numerical simulations were performed to optimize the geometric design of the microelectrodes configuration as well as to determine the applied electric field and the forces subsequently acting on pancreatic islets and exocrine tissue fragments. A custom electronic setup was developed for the generation of sinusoidal signals with proper voltage and frequency. Experiments on developed electrodes configurations were performed with single pancreatic beta cells and with samples of Langerhans

islets and pancreatic exocrine tissue fragments. Dielectric models of cells and islets were verified and negative DEP, showing repulsion from the electrodes, was observed for pancreatic islets. The results of this work demonstrate that Langerhans islet cells can be manipulated without physical contact by dielectrophoresis, a technique which can be applied on single cells as well as on cell aggregates in miniaturized lab-on-a-chip devices.

MULTIPARAMETRIC FLOW CYTOMETRY OF BRONCHOALVEOLAR LAVAGE FLUID CELL ANALYSIS BY SINGLE TUBE TEN COLOR

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Bronchoalveolar lavage (BAL) analysis in flow cytometry (FCM) is an important diagnostic tool in various diffuse lung diseases. Respect conventional immunochemistry, FCM immunophenotyping of lymphocytes in BAL samples provides results with excellent sensitivity and specificity. Using FCM BAL analysis CD4/CD8 ratio determination is reliable, precise and fast. Inflammatory cell differential counts in BAL can also be easily performed by multiparametric FCM, rather than with cytology on cytospin preparations, that is more time consuming and greather variability affected. The clinical utility of bronchoalveolar lavage fluid (BAL) cell analysis for the diagnosis and management of patients with interstitial lung disease (ILD) has been recently discussed by a committee of international experts of American Thoracic Society, but there are not FCM guidelines about antibody panels and gate strategies.
Between 2006 and 2012 we have performed more than 200 BAL fluid analysis by FCM and, from the last year, we perform them with a ten color multiparametric staining in a single tube, using Navios Cytometer (Beckman Coulter) and Kaluza software for analysis. To determine cell differential count we performe BAL cytospin by optical microscopy and BAL FCM labelling to detect cell differentiation and lymphocyte subsets. Usually we use a panel with monoclonal fluorochrome conjugated antibodies directed against CD3, CD4, CD8, CD9, CD14, CD15, CD19, CD45, CD(16+56) and HLA-DR. There is a good correlation between FCM and optical microscopy for differential cell count, although when it is performed by cytocentrifuge technique underestimates lymphocytes, particularly if their numbers are high. The technology development we allowed to improve the FCM method for BAL cell analysis. Our results show that FCM is feasible and reliable method to count BAL cell fraction based on cell antigen expression.

ANALYSIS OF CIRCULATING ENDOTHELIAL CELLS (CECs) AND ENDOTHELIAL PROGENITORS (EPCs) BY FLOW CYTOMETRY FOR CLINICAL PURPOSES: URGENT NEED OF A METHODOLOGICAL STANDARDIZATION

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Given the importance of vasculature in the biology of tumors (as well as in several diseases), also as a potential target for treatment, there is an increasing need for biomarkers of angiogenesis. Flow cytometric (FCM) detection of CECs and EPCs in whole PB is a fast assay to enumerate and characterize these cells in clinical studies, but remains a technical challenge. Their number is low and they cannot be characterized by a single marker but often discriminated using a combination of antigen with low, dull or a continuum of cell surface expression.
We have evaluated 20 clinical studies published from 2007 to 2013, based on the utilization of FCM. Each paper was analyzed for: FCM protocol, panels of endothelial markers, type of disease, number of pts and study design, overall clinical value ascribed to CECs and EPCs. Herein we summarized the pertinent findings which likely contributed to the differences observed across studies. The multitude of detection FCM methods applied (not always sufficiently detailed), togheter with the heterogeneity of the pt series, greatly limits comparability. A lack of a inter-laboratory standardized FCM assay and of a consensus on CEC and EPC phenotype has resulted in an enormous heterogeneity in the reported number of CECs and EPCs. Hence, even when looking at similar clinical conditions, the overall clinical value of the studies is partially limited.
In conclusion, although significant changes in the number of CECs and EPCs in different diseases have been reported and several steps forward the definition of the potential for clinical purposes have been achieved, their reliable identification and quantification still remains difficult. For clinical purposes, the interpretation of results should be made cautiously; the specificity of the antigenic combination for CEC and EPC definition and the inter-laboratories standardization of enumeration procedures are highly warranted to use these cells as clinical biomarkers.

A NOVEL APPROACH TO DETECTING RNA EXPRESSION IN LIVING BREAST CANCER CELLS

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When studying the complexities of cancer biomarkers, the ability to monitor gene expression within live cells that have become cancerous provides better insight than examining fixed or lysed tissues. However, monitoring RNA within intact cells can prove to be challenging with current techniques, which involve complex RNA isolation procedures that compromise cell viability. Further, amplification methods can create false positives or erroneously inflate differences. Here we describe a probe-based detection method that allows for the detection of ErbB-2 and EGFR mRNA levels across live intact SK-BR-3 breast cancer cells without the need for transfection reagents. The detection methodology is based upon an oligonucleotide gold nanoparticle conjugate capable of detecting intracellular levels of mRNA and miRNA in living cells. The probes enter the cell by means of the cell's native endocytosis machinery. Upon binding to the complementary target RNA sequence, a fluorescent signal (flare) is released, which allows the cells to be detected on any fluorescence analysis platform. In contrast to traditional RNA detection methods, since our technique allows for detection within live cells without altering their gene expression, the same cells that were profiled for

EGFR and ErbB-2 expression could be sorted based on their specific mRNA content and subsequently further studied. Stimulation or treatments could then be performed on the previously profiled cells and assessed using probes for additional RNA markers or more traditional techniques such as antibody staining, qRT-PCR, or array profiling. For this purpose, we combined immunostaining for cell surface EGFR with fluorogenic nanoparticle-based detection of EGFR mRNA in four breast cancer cell lines known to range in EGFR expression levels, and we observed significant correlation between mRNA and protein signals using two-color flow cytometry. Thus, detecting RNA expression levels in live cancer cells with the ability to utilize the same cells in downstream testing gives researchers the ability to perform experiments which were previously thought to be impossible.

GROUP I METABOTROPIC GLUTAMATE AUTORECEPTORS INDUCE ABNORMAL GLUTAMATE EXOCYTOSIS IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Glutamate-mediated excitotoxicity plays a major role in ALS and reduced astrocytic glutamate transport was suggested as a cause. Based on previous work we have proposed that abnormal release may represent another source of excessive glutamate. In this line, here we studied the modulation of glutamate release in ALS by Group I metabotropic glutamate (mGlu) receptors, that comprise mGlu1 and mGlu5 members. Synaptosomes from the lumbar spinal cord of SOD1/G93A mice, a widely used murine model for human ALS, and controls were used in release, confocal or electron microscopy and Western blot experiments. Concentrations of the mGlu1/5 receptor agonist 3,5-DHPG >0.3 μM stimulated the release of [^{3}H]d- aspartate, used to label the releasing pools of glutamate, both in control and SOD1/G93A mice. At variance, ≤0.3 μM 3,5-DHPG increased [^{3}H]d-aspartate release in SOD1/G93A mice only. Experiments with selective antagonists indicated the involvement of both mGlu1 and mGlu5 receptors, mGlu5 being preferentially involved in the high potency effects of 3,5-DHPG. High 3,5-DHPG concentrations increased IP3 formation in both mouse strains, whereas low 3,5-DHPG did it in SOD1/G93A mice only. Release experiments confirmed that 3,5-DHPG elicited [^{3}H]d-aspartate exocytosis involving intra-terminal Ca^{2+} release through IP3-sensitive channels. Confocal microscopy indicated the co-existence of both receptors presynaptically in the same glutamatergic nerve terminal in SOD1/G93A mice. To conclude, activation of mGlu1/5 receptors produced abnormal glutamate release in SOD1/G93A mice, suggesting that these receptors are implicated in ALS and that selective antagonists may be predicted for new therapeutic approaches.

PERFORMANCE EVALUATION OF A SIMPLE FLOW CYTOMETER IN HEMATOPOIETIC STEM CELL COUNT: A COMPARISON WITH A GOLD STANDARD INSTRUMENT

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Accurate estimation of cell counts by flow cytometry may be difficult in laboratories where sophisticated equipment and staff with specific flow cytometry expertise are not available. Relatively inexpensive Flow Cytometers (FC) able to perform basic functions may help to overcome these difficulties.
In this study we compared Hematopoietic Stem Cell (HSC) and leukocyte counts determined with the small and relatively inexpensive BD Accuri® C6 FC equipped with CFlow Plus® v. 1.0 software, with those obtained with the eight-color BD FACSCantoII® equipped with FACSDiva® v. 4.0.2 software, our gold standard instrument.
We tested in parallel 85 consecutive samples from cord blood, peripheral blood from onco-hematology patients, fresh and thawed HSC collected by apheresis and bone marrow products. All samples were analysed in duplicate. Results were compared with the one way ANOVA test, linear regression and Bland-Altman analysis. Result reproducibility was expressed as coefficient of variation (CV) of replicates. According to our procedures, CV exceeding 20% require test repetition.
Time for instrument setup, calibration and analysis was slightly longer with Accuri C6® (40 min) than with FACSCantoII® (30 min). Calibration of Accuri® C6 failed in about 20% of the experiments; a second calibration required further 15 minutes. Both HSC and leukocyte counts were highly correlated (r^2>0.99; P<0.0001) and did not differ significantly (p>0.05); the Bland–Altman analysis showed good count concordance with a moderate trend to larger deviation at low HSC counts (0-10/μl). Mean CVs of HSC and leukocyte count replicates were 7.1% and 3.3% for the Accuri® C6 and 4.9% and 3.1% for the FACSCantoII®, respectively, with no cases exceeding 20%.
In conclusion, Accuri® C6 is a reliable instrument for HSC and leukocyte enumeration. Its use is very simple and does not require deep flow cytometry knowledge. Its performance may be improved by a more efficient calibration and shorter analysis time.

3D CELL MICROINCUBATOR WITH INTRINSIC OPTICAL TRANSDUCTION CAPABILITY FOR ADVANCED CELL ANALYSES

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We have demonstrated that vertical high aspect-ratio photonic crystals can play the role of microincubators for cell culture in a three-dimensional (3D) environment and simultaneously the role of label-free optical detectors. The investigated microstructures, fabricated by electrochemical micromachining of standard silicon wafers, con-

sist in periodic arrays of 3 μm thick silicon walls separated by 5 μm wide, deeply etched air-gaps and feature the typical spectral properties of photonic crystals in the near infrared wavelength range 1.0 μm – 1.7 μm. Their spectral reflectivity, detected by means of a light beam traveling back and forth through silicon walls and gaps, is characterized by wavelength regions where propagation is forbidden and reflectivity is high (photonic bandgaps), separated by narrow regions where reflectivity is very low. As the presence of cells grown inside the gaps strongly affects light propagation across the photonic crystal and its spectral reflectivity, we have exploited this feature to directly probe the cell distribution with a label-free optical detection method. In particular, after demonstrating that human fibrosarcoma HT1080 cells are able to actively populate the deep, narrow gaps, growing adherent to the vertical silicon walls, we have used a fiberoptic setup for spatially mapping the variations of the line-shape of the reflected power spectrum due to the cell presence. Finally, we have shown that the intensity ratio at two specific wavelengths is well correlated to the cell spreading onto the silicon wall. These results represent the starting point for future *in-situ* label-free analyses of cellular activities (e.g., proliferation, differentiation and apoptosis) involving changes in cell morphology and/or adhesion, directly performed on a microstructure suitable for 3D cell culture.

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MESENCHYMAL STEM CELLS: BIOPHYSICAL CHARACTERIZATION AND OSTEOGENIC DIFFERENTIATION

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Human mesenchymal stem cells (hMSC) are able to self renew or to be committed towards cells of various lineages, such as osteoblasts. For this reason they have acquired a promising role in the field of regenerative medicine, even if major limitations to their clinical application to repair bone defects exist due to the poor availability of cells and the time required to differentiate to a stage suitable for implantation. Physical stimuli, such as pulsed electromagnetic fields (PEMFs), have been widely used in orthopedics and, at the moment, PEMF therapy is approved for bone disorders. The mechanism through which PEMF promotes the formation of bone remains however elusive. Our activity focused on the *in vitro* characterization of the molecular mediators of the effects of PEMF treatment on osteogenic differentiation of hMSC. In particular, through a combined biochemical and biophysical approach, we concentrated on the role of calcium, highlighting a potential role of intracellular calcium stores in the PEMF-induced differentiation pathway.

DRUG DELIVERY SYSTEMS: MULTI-WALLED CARBON NANOTUBES AS CARRIERS FOR MITOXANTRONE

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Carbon nanotubes (CNTs) have been introduced recently as a novel carrier system for both small and large therapeutic molecules. Most of the research on CNTs has focused on their potential for delivery of anticancer agents. In our work will discuss the efficacy of Mitoxantrone (antineoplastic drug, MTX) absorbed on multiwalled-CNT (MWCNTs) on human breast cancer cell line, MDA. MTX was adsorbed on oxidized MWCNTs by interactions with CNT carboxylic group and drug amine groups. The adduct has been characterized through Raman Spectroscopy, Thermo Gravimetric Analysis (TGA), and TEM. Trypan blue dye exclusion assay was used to investigate the cytotoxic effect of MTX adsorbed on CNTs. The results showed that Mitoxantrone loading on carbon nanotubes produced a reduction in MDA cell viability slightly higher if compared to MTX in solution. Cell viability in the presence of MTX loaded carbon nanotubes was dose- and time-dependent. These preliminary results showed that MWCNTs-MTX are as much effective as the free drug in killing tumor cell line; its physico-chemical and pharmacokinetics properties may support its use as an in-situ neo-adjuvant and/or adjuvant cytotoxic device. Actually, we are studying the combinatory effect of MWCNTs-MTX and some antineoplastic gallium salts, with the purpose of obtain a reduction of therapeutic MTX dose, maintaining drug's efficacy.

NEW SOURCE OF MULTIPOTENT MESENCHYMAL CELLS FOR TISSUE ENGINEERING STUDIES: WASTE HUMAN OVARIAN FOLLICULAR FLUID

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3D biomaterials have been widely used in reconstructive bone surgery as cell carriers providing mechanical support and as promoters of cell attachment and proliferation. In particular, gelatine cryogel scaffolds are promising new biomaterials owing to their biocompatibility and to substain the differentiation of mesenchymal stromal stem cells (MSCs). MSCs are multipotent stem cells that proliferate onto the surfaces with fibroblastic morphology and can differentiate into osteoblasts, chondrocytes and adipocytes. These cells can be isolated from several sources, including bone marrow and adipose tissue. Our

previously studies showed the possibility to obtain MSCs also from the human ovarian follicular liquid (FL) that is usually wasted during *in vitro* fertilization. In this study, our aim was to show the possibility to use FL cells combined to a promising biomaterials for tissue engineering in regenerative medicine. We tested the ability of these FL cells to grow and differentiate in minimal culture conditions on gelatine cryogel, in comparison with MSCs derived from human bone marrow. Samples and controls were analyzed with confocal and scanning electron microscopes. Results showed that mesenchymal FL cells on gelatine cryogel scaffold were able to adapt to surface structure of biomaterial scaffold, growing not only on the top surface but also in the layers below till 60 μm of deepness. Positive immunostaining for vimentin and CD44 (a typical MSC marker) of these cells on cryogel confirmed their mesenchymal stemness and demonstrated an excellent compatibility with the biomaterial surface. Bone marrow MSCs, used as control, showed a similar behavior. Preliminary results showed also the capability of FL cells on scaffold to be induced to osteogenic differentiation, producing bone extracellular matrix and expressing some specific proteins (i.e.osteopontin). In conclusion, MSCs derived from waste human ovarian follicular liquid showed promising affinity with 3D gelatine cryogel, opening new potential developments in biotech and medical applications.

MULTICOLOR IMMUNOFLUORESCENCE STAINING ALLOW DETAILED MORPHOLOGICAL STUDIES OF A549 LUNG ADENOCARCINOMA CELLS AFTER TGF-β1 TREATMENT

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Objectives: Here we investigate Cytoketatin (CK) and Vimentin (Vm) markers distribution in A549 lung adenocarcinoma cell line before and after TGF-β1 treatment and we propose our immunofluorecent staining protocol.
Methods: To investigate CK and Vm expressions, cells were plated in a 24well plate (P24w) in which round slides to allow the cells to grow above were placed. At 70% of confluence, cells were treated with 2ng/ml of TGF-β1 for 48 hours. Then, untreated and treated cells were fixed in 4% paraformaldehyde for 20 min at +4°C and permeabilized with 0.5% Triton for 5 min. A blocking step was performed with 5% BSA. The primary antibodies dilution was 1:150 for rabbit anti-Human Cytokeratin (Abcam) and 1:100 for mouse anti-Human Vimentin (Abcam), overnight at +4°C. The secondary antibodies diluition was 1:400 for goat anti-rabbit IgG DyLight594 (Abcam) and 1:500 for goat anti-mouse IgG FITC (ImmunoReagents, Inc.), for 90 min. at +4°C. The nuclei were counterstained with DAPI 300nM (LONZA). Multicolor staining were performed as following: DAPI-CK, DAPI-Vm, DAPI-CK-Vm. Isotype controls were used for each sample.
The samples were observed with Axioplan2 equipped with a HBO100 mercury vapor lamp and an AxioCam MRc5 camera, and analyzed using AxioVision Rel.4.8.2 software, equipped with the module Multidimensional Acquisition, all provided by Zeiss.
Results: The untreated and treated A549 cells expressed both CK and Vm markers. In untreated cells, IF analyses showed a homogeneous distribution of CK whereas Vm was localized in perinuclear vesicles in cytoplasm. After treatment, IF assay showed down-regulation of CK and Vm resulted to be distributed homogeneously to form intermediate filaments of cytoskeleton.
Discussion: Despite the problems in the multicolor staining protocol as the possible crossreactivity between the antibodies used, this staining allows you to make an accurate analysis of the distribution of makers analyzed on the same cell.
The simultaneous staining with the two markers in association with DAPI counterstaining, allows to appreciate the different localization of the markers around the nucleus of the cells in order to better understand the effect of TGF-β1, the main inducer of Epithelial-Mesenchymal Transition process, on remodeling of cytoskeleton.

A COMPARISON BETWEEN OPERATOR AND AUTOMATED SOFTWARE ANALYSIS OF FLOW CYTOMETRY DATA

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In the recent years, several software packages for the automated analysis of flow cytometry data have become available. Here, we aimed to compare the traditional operator-dependent analysis of T-cell subpopulations on multiple samples with the analysis performed by the Spanning-tree Progression Analysis of Density-normalized Events software (SPADE, Qiu et al., Nat. Biotech 2011, Bendall et al. Science 2011). Whole blood collected from 3 healthy donors was stained with fluorophore-conjugated monoclonal antibodies against CD3, CD4, CD8, CCR7 and CD45RA. After red blood cell lysis, samples were acquired and T-cell subpopulations were independently determined by an operator (using computer-assisted tools) or by means of SPADE. The data, expressed as the percentage of cells in the parent gate, were analyzed using statistics for method comparison. When all measurements were pooled, results showed a good agreement between the two methods: (bias: 0.8; r: 0,97; linear regression: y=0,6229+0,9949x). When the single T-cell subpopulations were analyzed, bias obtained was: total CD4: 0.4; CD4 naïve (n): 1.9; CD4 central memory (cm): 0.5; CD4 effector memory (em): -0.43. For CD8 T-cells bias was: total CD8: 0.4; CD8n: 0.2; CD8cm: 5.2; CD8em: 2.7; CD8emRA: -3.4. Again, these results showed a good agreement between the two methods for all T-cell subpopulations, except for CD8cm and CD8 emRA. This discrepancy could be due to the low number of measurements as well as the continuous and non distinct pattern of the two subpopulations. Our preliminary results suggest that the automated analysis is consistent with the operator-based approach. The implementation of the automated method could support routine cytometry data analysis, thus providing a robust and significantly more rapid means of extrapolating multi-dimensional data in multi-sample (longitudinal) studies.

A SIMPLE METHOD FOR THE TITRATION OF THE ISOTYPE-MATCHED ANTIBODY COULD BE USEFUL FOR A BETTER CONTROL OF THE BACKGROUND FLUORESCENCE

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The precise definition of the boundary between the positive (+ve) and the negative (-ve) cell population for those antigens displaying a continuos pattern of expression, remains one of the most discussed issues in flow cytometry. Several methods are used to minimize and control the background fluorescence to better define the +ve population, including antibody (Ab) titration, Fluorescence Minus-One controls (FMO) and Isotype-matched Controls (IC). FMO is the best procedure to determine the background fluorescence in the channel of interest, although does not provide information about the degree of non-specific binding of the Ab. To this aim, the combined use of FMO and IC might be useful to better determine the boundary between the +ve and –ve population. However, the use of manufacturer-recommended volumes of IC often results in an overestimation of the background fluorescence. Here, we address this problem showing a method to titer the IC for multicolor experiments. This method was used to determine the number of CD38+ve T-cells in 3 healthy donors (HD). First, the serially diluted IC phycoerythrin (PE)-conjugated and the titered CD38 PE-conjugated Abs were incubated with a fixed number of antibody-capture beads. IC titer was interpolated using IC dilution curve, from the point in which CD38 and IC had the same value of Median Intensity of Fluorescence. Results show that the numbers of CD4CD38 and CD8CD38+ve cells obtained using the titered IC are intermediate (median CD4CD38 for the 3 HD: 230cells/ul; median CD8CD38: 79cells/ul) between those obtained with the FMO control only (median CD4CD38: 258cells/ul; median CD8CD38: 100cells/ul) and with the untitered IC (median CD4CD38: 186cells/ul; median CD8CD38: 34cells/ul). This method could allow a more confident definition of the boundary between –ve and +ve cell population, in case of antigens following a continuous pattern of expression. In particular, it could be useful in the analysis of dimly expressed antigens.

Oncology

HUMAN PLACENTA-DERIVED NEUROSPHERES ARE SUSCEPTIBLE TO TRANSFORMATION AFTER EXTENSIVE *IN VITRO* EXPANSION

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Introduction: The cancer stem cell model links neoplastic cells with normal stem cell biology, but little is known on how normal stem cells are transformed into cancer stem cells.
Methods: To investigate the processes underlying the transformation of normal stem cells we developed *in vitro* a cancer stem cell model from human amniotic and chorionic placenta membranes. In this model we studied the expression of specific stem cell molecules by flow cytometry, and genes by real time RT-PCR. Microscopy immunfluorescence was employed to investigate the proliferative and differentiation patterns. Paired t-test analysis was employed for the statistical significance.
Results: Normal human stem cells from amniotic and chorionic placenta membranes differentiated into neural cell lineages, under specific conditions, to form secondary neurospheres with a capacity for self-renewal. After extensive *in vitro* culture, these cells underwent spontaneous transformations and acquired a neuroblastoma (NB)-like phenotype with an elevated proliferative potential that is comparable to established neuroblastoma cell lines. The ability of these cells to transform their phenotype was evidenced by an increased clonogenic ability *in vitro;* by augmented expression level of certain proliferation- and transformation-related genes (e.g., CCNA2, MYCN, ENPP2, GRIA3, and KIT); by the presence of multinucleated and hyperdiploid cells. We further demonstrate that the transformed phenotype is an NB by measuring the expression of an NB-specific cell-surface marker, disialoganglioside GD2.
Conclusions: We have developed a cancer stem cell model starting from normal human stem cells derived from amniotic and chorionic placenta membranes. These cells are able to differentiate into neural cell lineages and to undergo spontaneous transformations and acquire an NB-like phenotype.

EVIDENCES OF IMMUNOFLUORESCENCE ASSAYS USING FLUORESCENT SILICA NANOPARTICLES (NPs) BIOCONJUGATED

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Fluorescent antibodies are widely employed in many

immunofluorescence assays, but molecular fluorophore only used in bioconjugations are not particularly photostable and the intensity of the emitted light may be pH dependent or quenched by external agents.
Dye-doped silica nanoparticles (NPs) help to overcome these limitations, because the matrix acts as a protective shell against interfering species; moreover the molecular recognition event is signaled from all the dyes included in the nanoparticles. Silica also offers many advantages, being transparent to UV-Vis light, biocompatible, non toxic, inexpensive and extreme versatility.
Silica nanoparticles are fabricated and subsequently conjugated to antibodies.
The manufacture of our bioconjugates involves a preliminary activation of NPs with a specific cross-linker followed by the antibody modification using a reducing agent and the conjugation of the two species. The conjugation mixture is purified by Size Exclusion Chromatography and by Affinity Chromatography to achieve the maximum grade of purity. Our result show a great level of purity (about 96% of purity) because of the good separation between every single component of our conjugation bulk that we can easily isolate through the combination of two consecutive purifications.
Our dates suggests that the bioconjugated silica nanoparticles are optimal reagent for immunfluorescense assay as flow cytometry and CellSearch apparatus, a system to isolate, collect and quantify rare cells. The advantages of their application are a reduced background, better discrimination between different cellular population, high fluorescence emission, compatibility with standard staining procedure and analysis, high stability in the time.

HIGHER LEVEL OF TRANSFORMING GROWTH FACTOR-β1 IN TUMOR-DRAINING PULMONARY VEIN THAN PERIPHERAL BLOOD IN PATIENTS WITH NON SMALL CELL LUNG CANCER: A POSSIBLE MARKER OF TUMOR PROGRESSION

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Objectives: Transforming Growth Factor-β1 (TGF-β1) is a multifunctional factor that regulates cellular differentiation, proliferation, motility and is considered one of the main Epithelial-Mesenchimal Transition (EMT) inductors. Its has been found to be overexpressed locally in many tumors and it plays an important role in tumor transformation and progression. In order to investigate the possible role of TGF-β1 in patients with Non Small Cell Lung Cancer (NSCLC) as a marker of metastatic disease, we evaluated its level in plasma from the peripheral blood (PB) compared to that from draining pulmonary vein (RP), the blood vessel closer to the tumor site.
Methods: We analyzed the TGF-β1 plasma levels of 61 patients with NSCLC, enrolled from little more than a year. The TGF-□1 plasmatic concentration, expressed in pg/mL, was assayed by FlowCytomix-Human Basic Kit and the results obtained were analyzed by the FlowCytomix Pro program, all provided by eBioscience.
Results: In 25 of the 61 patients (41%) the TGF-β1 level from RP, wich ranges from 5,8ug/mL to 53,8ug/mL with a mean value of 23,4ug/mL, was higher than in the PB, which ranges from 5,2ug/mL to 37,1ug/mL with a mean value of 14,9ug/mL. In one of the 25 cases it was observed an increase in the concentration of TGF-β1 of more than 15 times of the RP compared to PB. No correlation with histopathological and clinical data was found.
Discussion: Metastasis is responsible for more than 90% of cancer associated mortality. For distant metastasis, primary tumor cells must invade, disseminate through blood vessels, seed at the distant site, and colonize to macrometastases. From our results no correlation with clinical data was found because all patients were enrolled from little more than a year but high levels of TGF-β1 right in the vessel proximal to the tumor (41% of patients analyzed) could be indicative of metastatic disease.

STUDY ON EXPRESSION OF HSA-MIR 17-3P IN HUMAN GLIOMA T98G CELLS TREATED WITH THE ANTI-OXIDANT DRUG GALLIC ACID

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miRNAs are small (22-25nt) non coding RNA molecules that are emerging as important regulators of biological processes. These molecules play a major role in post-transcriptional gene silencing and alterations in miRNAs expression are described in many different human diseases, including cancer. Several micro RNAs have been identified with potential importance in glioma biology and this may identify potential therapeutic microRNAs targeting functions relevant to both tumor biology and neurogenesis. It has been shown that gallic acid, one of the emerging candidate molecules with anti-cancer activity, contrasts tumor proliferation by suppressing angiogenesis, invasiveness and cell viability of U87 and U251 glioma cells *in vitro*. This drug is a basic component unit of tannic acid widely distributed in the plant kingdom with antiviral and antioxidant activity. In the present study, we studied the expression of hsa-miR-17-3p, a novel micro RNA that has been demonstrated to suppress tumorigenicity of prostate cancer and that inhibits mitochondrial antioxidant enzymes in human glioma T98G cells, with the aim to demonstrate a relationship between the anti-tumor effects of gallic acid and the down regulation of antioxidant activities. Our results demonstrated that gallic acid induces a progressive increase in cell death and in the expression levels of hsa-miR-17-3p with the increasing concentration of the drug (dose range: 1-100 ng/ml). On the contrary mitotic index is progressively significantly reduced. Considering these our preliminary results and that hsa-miR-17-3p is involved in many important signalling pathways (i.e. MAPK, neurotrophin, mTOR, Insulin) the use of gallic acid or its derivatives in the treatment of glioblastoma could represent the beginning of new promising therapeutic strategies.

ANTIANGIOGENIC POTENTIAL OF THE TELOMERE-TARGETING AGENT RHPS4: INSIGHT INTO THE MECHANISM OF ACTION OF A NEW CLASS OF ANTI-CANCER MOLECULES

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Angiogenesis, the process of new capillary formation from pre-existing vessels, plays an essential role in the embryonic and postnatal development, in the remodelling of various organ systems, and in several pathologies, including cancer. In the last context, it has long been appreciated that tumour growth and progression are dependent on angiogenesis and, on the basis of these results, a growing number of anticancer treatments – both conventional and novel targeted therapies – have shown to directly or indirectly suppress the neovasculature in established tumours.
Here, we have specifically investigated the effect on tumour angiogenesis of the pentacyclic acridine RHPS4, a telomere-targeting agent having antitumour activity through its ability to induce DNA damage. Our data clearly demonstrate that RHPS4 treatment induces a robust inhibition of cell migration, invasion and vessel formation. Moreover, the experiments performed on both human umbilical vein endothelial cells (HUVEC) and mouse polioma middle T transformed endothelial cells (H5V), demonstrate that RHPS4 acts at the level of monomeric GTP-ases of the Rho family (*i.e.* RhoA and Rac1) by negatively regulating the reorganization of actin cystoskeleton. Altogether these results reveal a novel, and so far undescribed, antiangiogenic effect of RHPS4 and suggest a peculiar role of this potential anticancer drug against those malignancies requiring neovasculaure for growth and spread.

THE METASTATIC POTENTIAL OF TUMOR CELLS CAN BE REVEALED BY 3D CULTURE ON A SILICON OPTICAL MICROCHIP

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Background. The metastatic process includes a series of steps in which cancer cells migrate or flow through different microenvironments (ME). The ability to successfully originate a secondary tumor is partially dependent on the physical interactions and mechanical forces between cancer cells and the ME. In this process, in particular, cells must undergo large elastic deformations. A better understanding of the role of biophysical and mechanical properties of cells will provide new and important insights into the progression of cancer and could provide the basis for new diagnostic/prognostic approaches. We investigated the cell membrane plasticity as an indicator able to forecast the tumor cell behavior with regard to mobility and/or migration. Both these actions are strongly related to the cell metastatic potential (MP).
Methods. Silicon micromachined structures (SmS) were exploited as 3D microincubators able to host tumor cell lines with different level of MP. The 3D microstructures selected for this research consist in periodic arrays of silicon walls, with thickness of a few μm, separated by empty gaps (width of 5 μm), and high vertical aspect-ratio (height up to 100 μm), fabricated by electrochemical micromachining of silicon dice. After incubation (24-48hrs) SmS samples were washed in PBS and fixed overnight in cold 70% ethanol and later stained with FITC/PI for fluorescence microscopy. Cell culture experiments were performed in triplicate on 4 different established cell lines, two of them with high MP (MDA-MB-231, RPMI-7951) and 2 with low MP (MCF-7, CAPAN-1). Cells from the K562 line were used as the negative control.
Results/Conclusions. Fluorescence microscopy of SmS populated by cells clearly evidenced the following behavior: a) K562 cells were found only on the surface of SmS; b) low MP cells are largely unable to grow on the SmS surface and just a few cells can survive on the top of the walls (their nuclei appear to have round shapes typical of cells growing on flat surfaces); c) high MP cells can grow on the SmS thanks to their ability to colonize the narrow empty space between silicon walls (red stretched nuclei all aligned along the wall direction clearly prove that these cells are deeply inside the extremely narrow gaps of the SmS). The less stiff (more "plastic") cytoskeleton/membrane of this second type of cells (more metastatic) corresponds to their bio-mechanical ability triggering their "clinical aggressivity" also exhibited on SmS: these structures thus become an interesting candidate to be implemented in a lab-on-chip for clinical testing.
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THE *MET* ONCOGENE IS A FUNCTIONAL MARKER OF A GLIOBLASTOMA STEM CELL SUBTYPE

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The existence of treatment-resistant cancer stem cells contributes to the aggressive phenotype of glioblastoma. However, the molecular mechanisms that sustain stem cell proliferation in these tumors are largely unknown. We report that expression of the MET oncogene, encoding the HGF Receptor, identifies a subset of glioblastoma-derived neurospheres (MET-pos-NS), characterized by a gene expression profile classified as *mesenchymal* or *proneural*. Conversely, neurospheres that do not express MET (MET-neg-NS) usually harbor an amplified EGFR gene and display a gene expression profile defined as *classical*. We show that MET-pos-NS are hierarchically organized, and include a cell subpopulation expressing high levels of MET (MET-high) at the top, and a cell subpopulation that conversely express negligible levels of MET (MET-low), at the base. This conclusion is supported by data showing that, upon flow cytometric isolation from neurospheres, (i) MET-high, but not MET-low, cells retain long-term propagation and multi-potential differentiation; (ii) MET-high, but not MET-low, cells can regenerate a mixed MET-high/MET-low cell population; (iii) MET-high cells display increased tumorigenic ability as compared to MET-low cells, and are the only that form tumors containing both MET-high and MET-low cells. In MET-high cells, we also find that HGF sustains the stem cell phenotype (expression of self-renewal markers, clonogenicity and invasion) in vitro. Together, these findings suggest that MET is a functional marker of glioblastoma stem cells, and a promising target for identification and therapy of a subset of glioblastomas.

CANCER STEM CELLS IN HEAD AND NECK TUMORS: EVIDENCE FOR METASTATIC SPREAD AND TREATMENT RESISTANCE

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The major challenge in the management of patients with oral squamous cell carcinomas (OSCC) is the development of resistance to therapy leading to disseminated disease. Since cancer stem cells (CSC) have emerged as important players in OSCC metastasis, our objectives were to explore the implications of CSC in OSCC tumor progression, invasion and response to conventional therapies. Methods: A panel of well-characterized cell lines originated from the most common sites in the head and neck area was used. Cells were cultured as spheres or under normal adherent conditions and analyzed for CD44, ALDH, CD24, CD29, CD56 by flow cytometry, PCR arrays for genes related to stemness, metastasis and EMT. We also investigated sLeX expression, known to play a key-role in many cancers metastasis by promoting tumor cells binding to endothelial E-selectin. We analyzed the tumorigenic potential of OSCC cells by invasion assays and in vivo OSCC experimental models comparatively to CSC cells. Resistance to cisplatin and radiation was assessed by annexin V/PI assay and colony forming assay. Results: The highest levels of sLeX expression were found in cell lines originated from oral cavity (9%-47%) compared to other head and neck locations (0.1%-7%). Cells grown as spheres were 95-100% positive for sLeX compared to 10-40% of adherent counterpart. Although sLeX+ and sLeX- cells were both able to form spheres, sLeX+ spheres were predominant and larger. Flow cytometry and PCR arrays indicated that the spheres were highly enriched in CSC and metastatic markers. Consistently, the spheres showed increased invasive and tumorigenic potential, and resistance to conventional chemotherapy and radiations. Conclusion: this study is the first to unveil a novel link between sLeX expression, stem cell formation and metastatic spread in OSCC, and provides supportive evidence for CSC resistance to treatment. Understanding the mechanisms of tumor invasion and metastasis will improve patient outcome and survival.

MULTIPLEX FLOW CYTOMETRY IDENTIFICATION OF SURFACE SPECIFICITIES OF PRIMARY AND METASTATIC COLON CANCERS

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Identification of tumor associate antigens (TAA) is fundamental for prognostication, early detection and targeting of cancer cells. Cancer associate surface antigens can be also important target for molecules and drugs systemically delivered, functioning through the block of signaling pathways or antibody-dependent cell- and complement-mediated cytotoxicity. Flow cytometry allows the analysis of protein expression *in situ* and at single cell level. We analyzed, by multiplex flow cytometry, the expression of 27 surface antigens comparing their expression in normal colon, colon cancers and hepatic metastases. We determined a specific surface signature of colon cancer cells identifying putative functionally important proteins whose expression was significantly higher in primary tumors from metastatic patients as compared to non metastatic ones, as well as in metastasis samples as compared to primary tumors. We first compared the 27 antigens expression in normal colon and in colon cancer tissue. The expression level of 16 antigens (CD133, CD24, CD29, CD44, CD47, CD49b, CD49f, CD54, CD55, CD59, CD66b, CD66c, CD81, CD164, CD166, CD227) was significantly ($p<0.05$) enhanced in cancer samples respect to normal ones. We also observed an expansion of CD133, CD24, CD55, CD66c, CD221, CD227 expressing cell populations in tumor tissues respect to normal colon. Additionally, hepatic metastases had a significantly higher expression of CD24, CD26, CD66c and CD151($p=0.0099$, $p=0.0180$, $p=0.0119$, $p=0.0395$ respectively). We also analyzed the expression of the 27 markers on CD133+ and CD133- subsets of cancer samples to evaluate differences between the stem-like compartment and the more differentiated one. The expression level of 15 antigens was significantly ($p<0.05$) enhanced, in primary tumors, in CD133+ cells respect to CD133- ones. The stem compartment from hepatic metastases revealed significantly ($p=0.0062$) higher expression of the integrin CD29. Finally, one of the differentially expressed antigens: CD26/DPPIV, was found on circulating cancer cells from peripheral blood of colorectal cancer patients. The presence of the CD26+ circulating cells strongly correlated with cancer recurrence for which it was a prognostic factor in multivariate analysis.

MYCN GENE EXPRESSION IS REQUIRED FOR THE ONSET OF DIFFERENTIATING PROGRAM IN CELLS OF NEURAL ORIGIN

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The *MYC* family gene, *MYCN*, plays a role during the first steps of human development. It is widely accepted that *MYCN* decreases during differentiation in cells of neural origin. We show that *MYCN* expression increases in embryonic cortical neural precursor cells after few days from differentiation induction. We used human neuroblastoma cell lines as model to investigate the role of *MYCN* in the earlier stages of neuronal differentiation. In LAN-5 neuroblastoma cells, *MYCN* is up-regulated after 2 days of retinoic acid treatment before its expected down-regulation. A positive modulation of some differentiation markers, such as *CDK5, ENO2, SLC18A3, MAPT/TAU, GAP43* and *ChAT,* is associated with the increase of *MYCN* expression. Likewise, *MYCN* silencing inhibits differentiation in LAN-5 *MYCN* knock-down (KD) clones, leading to a negative modulation of the aforementioned developing markers. In addition, the LAN-5 *MYCN* KD clones show a loss of neurofilaments formation and a depolarization of the plasma membrane. Furthermore, overexpression of *MYCN* gene in the poorly-differentiating neuroblastoma cell line SK-N-AS restores the expression of differentiation-related genes as well as neuritis outgrowth. NF200 immunostaining reveals an increased ability to form mature neurofilaments in SK-N-AS *MYCN*+ clones, after retinoic acid treatment. MicroRNA Assay analysis revealed that some specific miRNAs (miR9, miR20a and miR92a) are conversely modulated during

the silencing and overexpression of *MYCN* gene in the two different cell lines, indicating a possible role of these non-coding RNAs in driving the onset of differentiating programs.

A POSSIBLE ROLE OF LAMIN A/C IN ChAT-ACTIVATED DIFFERENTIATION MOLECULAR MACHINERY

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The mouse neuroblastoma N18TG2 clone is defective for biosynthetic neurotransmitter enzymes; thus appearing always as an immature neuronal clone. The forced expression of ChAT in these cells results in the synthesis of endogenous ACh and in their higher ability to express neurospecific features and markers. We investigated more in detail the wide genome expression profiling differences between the N18TG2 clone and its ChAT-transfected derived clone (2/4). The developmental program switching on in the 2/4 clone was confirmed by the increasing expression of several differentiation-related genes (CDK5, CEND1, Syn1, Syn2, Nav1, Tubb3) and by the decrease of typical cell cycle-related genes expression, such as Cyclins and Cdks. We also observed an increase of LMNA gene expression in 2/4 clone; interestingly, several studies demonstrated Lamin A/C shows a differentiation-related accumulation. In fact, we stimulated the differentiation process with retinoic acid and we observed an increasing expression of Lamin A/C protein in both clones during differentiation stimuli. We performed agonist/antagonist receptor study to establish if LMNA expression was strictly related to AChR activation: carbachol treatment (AChR agonist) led to increasing LMNA as well as neuronal differentiation markers expression in N18 cells; nevertheless, treating 2/4 clone with atropine or mecamylamine (AChR antagonist) caused a diminution in LMNA mRNA levels and a concomitant decrease of differentiation related genes. Our hypothesis is that Lamin A/C might be involved in the ChAT-dependent molecular differentiation pathway, hence we are trying to corroborate this putative role by silencing LMNA gene in 2/4 clone.

TGFβ1 HIGHLIGHTS SEVERAL SUBPOPULATIONS WITH DIFFERENT CHARACTERISTICS OF STEM CELL THAT LEAD TO METASTASIS

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Objectives: Metastasis is the leading cause of death by cancer. Non-small-cell lung cancer represents nearly 85% of primary malignant lung tumours. The epithelial-to-mesenchymal transition (EMT) plays a key role in metastasis. TGFβ1 is the major inductor of EMT. Aim of this study was to investigate the role of TGFβ1 on cancer stem cells (CSCs) and non-CSCs starting from A549 lung cell line.
Methods: A549 cell line was sorted for CD133 surface antigen and Side Population (SP) profile by flow cytometry. A549 cell fractions obtained were treated with TGFβ1 for 48 hours. After incubation, were analyzed by immunofluorescence, RT-PCR and Western Blot. Migration, Wound-healing were performed to assess the migratory capacity. Soft agar assay was performed to evaluate tumorigenicity.
Results: TGFβ1 induced EMT in all fractions of A549 sorted. It increases the percentage of cells migrating except for $CD133^-$ fraction. The migration of SP^- cell fraction is stronger than of its untreated corresponding cells. Although this, $CD133^+$ cell fraction responded more effectively to the TGFβ1 treatment. For stemness, TGFβ1 induced an increase of OCT4 in all treated fractions except for $CD133^-$. Wound size reveals that TGF-β1 enhances motility in all cell fraction except for $CD133^-$ and SP^- cells. Assessment of growth kinetics reveals major colony-forming efficiency in $CD133^+$ A549 cells. For $CD133^-$ cells no change in colony number was observable.
Conclusion: The results obtained suggest that TGFβ1 highlights different stemness subpopulations with different characteristics: i) a stationary CSCs subpopulation identified as SP^+ cell subset and ii) a migratory CSCs subpopulation identified as $CD133^+$ cell subset. Each population seems to be involved in different biological mechanisms such as stemness maintenance, tumorigenicity, invasion and migration. Noteworthy and interestingly, also SP^- showed strong migratory capacity indicating TGFβ1 could induce cell reprogram on non-CSCs by EMT leading to coexistion by switching between the two phenotypic states.

ABSENCE OF LAMIN A/C AS POSSIBLE MARKER OF CANCER STEM CELLS IN NEUROBLASTOMA

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A-type lamins, major constituents of the nuclear lamina, are absent in most types of stem cells. In a previous paper we have shown that silencing of lamin A/C blocked differentiation in neuroblastoma cells. Neuroblastoma may be considered as a heterogeneous population of cells in which only a minor population capable of tumor initiation: the cancer stem cells. Our aim is to study the role of A-type lamins in the development of neuroblastoma as stem cells disease. We used the SH-SY5Y neuroblastoma cell line. Silencing of lamin A/C causes an increase of the expression of the stemness markers such as *CD133*/Prom-1. *CD133*+ cells were isolated from *LMNA*-KD cells using magnetic cell sorting technology. The *CD133*(+)-enriched cell population showed an increased expression of the stemness markers. The acquisition of a stem cell-like phenotype is also supported by the presence of a "side population". Consistently, the *LMNA*-KD cells expanded as non-adherent secondary sphere-like cellular aggregates in serum-free media containing EGF and FGF2. When tumour spheres were cultured without EGF and FGF-2 in the presence of 10% serum floating cells attached to the plastic as monolayer. This cell line retains the same stem cell phenotype. In the *LMNA*-KD cells, miRNAs expression profiling evidenced the modulation of a different number of miRNAs involved in differentiation, such as miR-124 and miR-101. Down regulation of miR-101 is consistent with the increased

levels of *MYCN* in *LMNA*-KD cells. We hypothesize that A-type lamins are necessary to maintain low levels of *MYCN* by regulating miR-101 expression.

EXCITOTOXIC DEATH INDUCED BY GLUTAMATE IN RAT CEREBELLAR GRANULE CELLS IS INHIBITED BY SILENGING LAMIN A/C

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The cerebellum granule cells (GCs) are the most numerous neurons of the entire central nervous system and lie within the internal granular layer, located below a monolayer of Purkinje cells. The maturation and differentiation of GCs is promoted by excitatory amino acids receptors activation, such as glutamate. However, an excessive activation may compromise the cell survival, this phenomenon (excitotoxic effect) being involved in many neurodegenerative diseases. The GCs provide an excellent experimental model for molecular and cell biological studies of neuronal development and neurodegenerative diseases, because their development *in vitro* mimics very well that *in vivo,* thus preserving the same mechanisms of glutamatergic fibers formation occuring during development. Primary cultures of post mitotic cerebellar GCs represent a homogeneous cellular system showing a single neuronal population since glia expansion is prevented by inhibiting mitosis. The primary cultures of GCs have a short life, in fact as their maturation progresses become gradually more and more vulnerable to the effect of glutamate, and after eight days of culture they reach their full maturity and die by necrosis. Our aim is to study whether A-type lamins, know to play a crucial role in the differentiation, can influence the granule neurons maturation. By immunohistochemistry, we have seen that Lamin A/C expression increases during the development of GCs *in vivo*. We monitored neurons maturation *in vitro* studying the cell response to stimulation by glutamate. Silencing of *LMNA* gene inhibited glutamate-mediated spontaneous neuronal death. Thus suggests that the *LMNA* knock down in GCs exhibits a sort of glutamate-resistant phenotype, which has been characterized by performing genome wide profiling.

EFFECTS OF GALLIC ACID ON VIABILITY, PROLIFERATION AND INVASION IN HUMAN GLIOMA T98G CELLS

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Glioblastoma multiforme (GBM) is the most common and most malignant primary brain tumor in adulthood. Despite treatment efforts including new technological advances in neurosurgery, radiation therapy, and clinical trials with novel therapeutic agents, the tumor recurrence is very high. Therefore, it is of critical importance that new effective treatments for glioblastoma must be developed. Gallic acid, an organic acid found both free and as part of , has been shown to inhibit carcinogenesis in animal models and *in vitro* cancerous cell lines. Previous studies have demonstrated that this molecule inhibits cell viability of U87 and U251 glioma cells in a dose-dependent manner. As such, the aim of this study is to evaluate the effects of gallic acid, in T98G human glioma cell line, , which it is known to have a high invasion ability and to be chemoresistant. We have performed viability, proliferation and invasion tests using five different concentrations. Our data demonstrated that gallic acid reduces glioma cells proliferation decreasing the mitotic process, but only high concentrations (100µg/ml) of gallic acid are able to induce cell death. We assume that T98G cells are more resistant than other glioma cell lines to gallic acid treatment because we obtained data similar to the literature only after three time higher concentrations of the drug. In conclusion gallic acid may be a valuable candidate for treatment of brain tumour in association with other more cytotoxic treatments.

ANALYSIS OF ANTITUMOR ACTIVITY OF TRABECTEDIN IN MYELODYSPLASTIC/MYELOPROLIFERATIVE NEOPLASMS

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Juvenile myelomonocytic leukemia (JMML) and chronic myelomonocytic leukemia (CMML) are myelodysplastic/ myeloproliferative neoplasms (MDS/MPN) of childhood and elderly patients, respectively. Current treatment options for JMML and CMML are highly unsatisfactory and the only curative therapyis allogenic BM transplantation. There is clearly a need to identify new effective treatment strategies for these patients. Trabectedin, a new DNA binder of marine origin was recently found to cause selective depletion of monocytes in blood and spleens of tumor-bearing mice, to activate caspase-8-dependent apoptosis, and TRAIL receptors expression. The aim of this study was to characterize the in vitro cytotoxic effect, the cell cycle phase perturbations, the expression of TRAIL receptors and the mechanism of cell death induced by trabectedin on biphenotypic B-myelomonocytic leukemia MV4-11 cell line and on the malignant myelomonocityc cell compartment of patients suffering from CMML or JMML.
As observed on MV4-11 cell line, in the first 20 cases we found that malignant monocytes from both JMML and CMML are highly susceptible in vitro to trabectedin compared to azacytidine and cytosine arabinoside (ara-c). Trabectedin, at nanomolar concentrations, was able to induce strong apoptosis, evaluated by flow cytometric annexin-V/PI assay, both on MV4-11 cells and on malignant monocytes from patients after 24, 48 and 72h treatment. On MV4-11 cells, apoptosis was found to be caspase-8-dependent. Trabectedin, on MV4-11 cells, induced the expression of TRAIL receptors, evaluated by flow cytometry, after 24 h treatment at the concentration of 0.5 nM, while in malignant monocytes from JMML and CMML did not. In addition, trabectedin inhibited in a dose dependent manner the clonogenic growth of myelomonocytic progenitors of CMML patients and the spontaneous growth of circulating CFU-GM of JMML patients, respectively. Molecular studies on malignant monocytes from

JMML and CMML treated with trabectedin are in progress to identify molecular pattern associated to clinical outcome. All togehther, our data suggest that Trabectedin could be a candidate for a new effective treatment in MDS/MPN, and in particular in JMML and CMML.

GENE EXPRESSION PROFILING OF SPHERES DERIVED FROM HUMAN PRIMARY CHONDROSARCOMA

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Background and Purpose: Solid tumours are made of cell subsets defined cancer stem cells with stemness potentials, aggressiveness and increasing expectancy of recurrence. These cells are able to form spheres. Aim of this study was to obtain chondrospheres from a primary chondrosarcoma and to compare the molecular and cellular characteristics with corresponding adherent cells.
Procedures: Primary chondrosarcoma cells were cultured both in standard and sphere culture condition. On both spheres and adherent cells, cytometric analyses for stemness and differentiation antigens including CD133, OCT4, Sox-2 and collagen type II were performed. In addition, cell cycle analyses and apoptosis assay were performed after cisplatin treatment performed for 48 h at different and high concentrations. Gene expressing profile microarray was performed.
Findings: Chondrospheres were formed already after 24 hours of culture. They over-expressed CD133, OCT3/4, and Sox2 markers and showed resistance to cis-platin treatment. Microarray analyses pointed out a huge gene expression difference between chondrospheres and adherent cells. With a cut-off threshold of 2-fold, 1405 genes were differentially expressed of which 629 genes were over-expressed and 776 under-expressed on chondrospheres. The most highly over-expressed genes were involved in multidrug resistance, cell cycle, apoptosis regulation, migration, motility and invasion regulations and stemness phenotype.
Conclusions: This study highlights that chondrosarcomas are a source of cancer stem cells and chondrospheres may constitute an excellent model to study stemness, tumoral heterogeneity and multi drug resistance to individuate candidate biomarkers for novel therapeutic targets.

SIDE POPULATION CHARACTERIZATION IN HEPATOCELLULAR CARCINOMA CELL LINES

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Side Population (SP) was initially identified as a population of hematopoietic stem cells able to extrude from their cytoplasm different drugs and the fluorescent dye Hoechst 33342 through the activity of ATP-Binding Cassette (ABC) proteins. More recently, SP was also found in several tumor cell lines with stemness and chemoresistance properties.
The aim of this study was to characterize SP in different HCC cell lines by means of the expression of ABCG2 and other ABC pumps and stem cell genes such as NANOG and OCT4.
Flow cytometry evaluation of SP and non-SP cells and ABCG2 membrane expression were performed in HCC1, Hep3B, HepG2, HuH7, SNU475 and PLC/PRF/5 cell lines following standard protocols. Real Time-PCR (RT-PCR) of ABCG2, ABCB1, ABCC1, MVP, NANOG and OCT4 was carried out on SP and non-SP cells, sorted by BD FACSAriaII for mRNA quantification. The t test was used to compare distibutions.
Side population was detected only in HCC1, Hep3B, HepG2 and HuH7 cell lines (16%, 3.2%, 0.3% and 0.2%, respectively). Only 4% of SP-HCC1 expressed ABCG2 on their membrane, in accordance with the results of RT-PCR confirming low levels of ABCG2 expression on SP-HCC1. Interestingly, SP cells of all the four cell lines expressed higher levels of ABCB1 as compared to non-SP ($p<0.011$}). For the other ABC proteins tested, no significant differences were found between SP and non-SP cells. With regard to stemness genes, only SP-HepG2 cells expressed higher levels of NANOG and OCT4 as compared to non-SP cells1} ($p<0.002$).
In conclusion, SP is detectable in several HCC cell lines and it is characterized by a high expression of ABCB1 rather than ABCG2. The upregulation of NANOG and OCT4 in SP-HepG2 cells supports the hypothesis that these cells have stemness properties. Further studies are needed to elucidate the role of ABCB1 in HCC chemoresitance.

MONOCLONAL ANTIBODY-BASED CANCER THERAPY AND DEVELOPMENT OF TUMOR ANTIGEN MIMOTOPES AS VACCINE

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The UN1 monoclonal antibody (mAb) was produced on the basis of selective reactivity with human thymocytes. We identified the antigen recognized by the UN1 mAb (UN1 Ag) as CD43 glycoforms that were peculiarly expressed in fetal tissues and several cancer tissues, while unexpressed in normal tissue counterparts. Based on the cancer-associated expression, the UN1 Ag was an attractive target for cancer immunotherapy.
In this study, we describe that the parental administration of the UN1 mAb delayed the tumor growth of UN1-positive T-lymphoblastoid HPB-ALL cells upon mouse engraftment. Based on the screening of an M13 phage-displayed random peptide library (RPL) using the UN1 mAb as bait, we selected UN1-like phagotopes that specifically bound to the UN1 mAb. We demonstrated that the mice immunization with a selected UN1 phagotope arose antibodies that specifically recognized the UN1 antigen in tumor tissues as well a synthetic peptide mimicking the UN1 epitope. Taken together these results indicated that the UN1 mAb: (1) was able to target in vivo cancer-associated CD43 glycoforms and inhibit the growth of tumor xenograft in mice, and (2) was an useful tool to select UN1 mimotopes from RPLs as potential cancer vaccine candidates

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