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EDITED BY
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XXVII National Conference of the Italian Society of Cytometry GIC

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Following the first experience in 2005, also this year an issue of Cytometry is partly dedicated to the programme and abstracts of the National Conference of the Italian Society of Cytometry, GIC. The XXVII edition of the Conference has been organized in October 2009 in Ferrara City, Italy. From 1995 on, UNESCO has included the historical centre of Ferrara in the list of World Cultural Heritage as a wonderful example of a town planned in the Renaissance and still keeping its historical centre intact. Its beauty has been linked to one of the most important courts in the political scenario of the 15th-16th century: the Estense court, which was one of the major actors in that precious season we call the Renaissance period.

As far as the GIC meeting is concerned, we want to stress the fact that all abstracts were carefully reviewed by the Scientific program Committee and published here in full and categorized by scientific track (1. cell cycle and apoptosis; 2. environmental sciences and toxicology, 3. hematology, 4. immunology, 5. methodology and technology, 6. oncology).

Following a continuous growth in these years, to date there are over 850 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 year, a great number of abstracts (>100) have been selected by the Scientific Committee among those submitted by basic and clinical researchers operating in the various Italian Institutions.

Each session involved invited lectures and was focused on the emerging role of cytometry techniques in Hematology, Stem Cell Biology, Immunology, Oncology and Environmental Sciences and Toxicology.

In addition, different topics of general interest in biological and medical sciences, new data on the study of a rare disease such as PNH, accreditation, standardization of ZAP70 measurement across Italy, and on the Methodological and Technological advances were reviewed by experts from Italy. Two of these lectures were dedicated to the loss of two "top" scientists, Prof Bruno Rotoli (Naples) and Prof Antonio Tabilio (Perugia). Both of them tirelessly helped young researchers and research students, and they were active in disseminating research findings to and communicating with the public. We do all miss them!

The Conference had been also characterized by a round table dealing with the possible interactions between parental scientific Societies having different levels of interest in cytometric techniques and applications Since many years ago the GIC Society did promote such kind of scientific interactions.

A substantial contribution was obtained from the principal industries in the field that have been located in a large exhibition area inside the conference center.

This national event is growing each year and, once again, represents Italian cytometry's scientific contribution to the international community.

Guest Editors:
R. De Vita - G. Mazzini

Francesco Lanza
GIC President

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INVITED SPEAKERS

THE EVOLUTION OF IMMUNE SYSTEM

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The Immune System in humans and higher vertebrates is an extremely complex array of cell types and molecules that interact among themselves, with foreign pathogens and with components of the own organism in multiple finely regulated processes. The advantages of our evolved immune system seem to focus in the sensitive definition of the self and the subsequent recognition of non-self elements that may menace survival of individuals and the species. The complexity of immune responses has been rather simplistically, but operatively, separated into two complementary branches, innate and adaptive immunity. Adaptive immunity is unique to vertebrates, while innate immune response have common features in vertebrates, invertebrates and even plants, including receptors for microbe-associated molecules, conserved intracellular signal transduction pathways and antimicrobial peptides. A clonally diverse anticipatory repertoire in which each lymphocyte bears a unique antigen receptor is the central feature of adaptive immunity. A key evolutionary event occurred half-a-billion years ago, when two types of recombinatorial adaptive immune systems appeared in vertebrates, associated to a stringent, darwinian-like, selection of the resulting cells. Jawed vertebrates generate a diverse repertoire of B and T cell antigen receptors through the rearrangement of immunoglobulin V, D, and J gene fragments, whereas jawless fish assemble their variable lymphocyte receptors through recombinatorial usage of leucine-rich repeat (LRR) modular units. In this presentation, the main molecular, cellular and taxonomical milestones of immune evolution will be summarized, together with the methodological approaches used to obtain such evidences and the practical applications of this knowledge. Finally, the opposing points of view regarding divergency or convergency of immune evolution and of the final benefits of the strategy adopted by the human immune system will be commented.

RESPONSE THERAPY IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA: THE ROLE OF FLOW CYTOMETRY

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Response to therapy in children with diagnosis of Acute Lymphoblastic Leukemia (ALL) has demonstrated to be a strong and independent prognostic factor with clinical impact, and can be measured by applying sensitive methods able to determine levels of minimal (submicroscopic) residual disease (MRD). MRD level can be influenced by several factors such as intrinsic biologic characteristics of the leukemic clone, bone marrow microenvironment, individual clinical features and pharmacodynamic/pharmacocynetic features of drugs. The overall influence of these variables determine patient's outcome. MRD measurement is currently applied in several clinical trials to assign patients to different risk categories during front-line treatment by using either Real Time Quantitative Polymerase Chain Reaction (RT-PCR) of receptor gene rearrangements or flow cytometric detection of leukemia-associated immunophenotypes. Flow cytometry is usually less expensive and faster than RT-PCR and has been largely standardized in both single and multi institutional studies, contributing to improve strategies of risk assessment in the management of children with ALL.

RECENT ADVANCES IN MOLECULAR CYTOLOGY AND THEIR APPLICATION IN TOXICOLOGY

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The development of new tools and technologies to visualize molecules at a cellular and subcellular level has greatly expanded the possibility to characterize the mode of action of potentially mutagenic and carcinogenic agents. Recently, the study of the so called DNA damage response (DDR) is becoming more interesting than the measure of DNA damage itself. The cellular heterogeneity of many target tissues and the stochastic nature of chemical-cell interactions often make less sensitive and informative the analyses on bulk tissues than those of histological sections. Immunocytochemistry to detect specific proteins and protein covalent modifications, or in situ hybridization to detect specific messenger RNA can be applied to visualize molecular changes in histological sections. Also multiparametric flow cytometry can be usefully employed to investigate the cellular response at a single cell level.

In the presentation, examples from the most recent literature will be shown in various experimental models and target organs, illustrating applications of molecular cytology to the study of the cellular response to stressing conditions: the detection and quantification of proliferating cells by labelling the proliferating cell nuclear antigen (PCNA) in fish intestine or rat testis, the detection of DNA damage signalling in mouse central nervous system or testicular cells

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by immunohistochemical analysis of ATM or γ-H2AX, the detection of DNA damage by immunolabelling of oxidized bases, the flow cytometric detection of chromatin remodelling as an early step of the DNA damage response.

MOLECULAR CYTOGENETIC LESIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA

Cuneo A, Cavazzini F, Ciccone M, Dabusti M, Cibien F, Daghia G, Sofritti O, Viglione GM, and Rigolin GM.

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In the 90's, only approximately 50% of CLL could be shown to carry a chromosome defect, a figure reflecting inadequate cell division. The introduction of FISH allowed for the detection of chromosome aberrations in 80% of the cases and every patient could be included in a specific group according to a hierarchical cytogenetic classification as follows: 17p- > 11q- > +12 > 13q- > normal. In most studies, approximately 40% of CLLs were shown to carry isolated 13q-, 10-15% of the patients carried +12 or 11q-, 2-5% 17p- or 6q- or 14q32 translocations. The variable incidence of specific lesions in different phases of the disease reflects their correlation with biologic and clinical features.

Recently, the introduction of effective mitogenic stimulation by oligonucleotides and interleukin-2 (IL-2) showed that approximately 30% of CLL without chromosome defects by interphase FISH carried a chromosome lesion by CBA in regions not covered by the FISH panel of probes. Complex karyotypes could be documented in a substantial fraction of cases in association with unfavorable prognostic factors and inferior clinical outcome.

In conclusion, molecular cytogenetic analysis in CLL revealed a number of lesions having important clinicobiologic implications. A fraction of CLL may acquire clonal chromosome changes during the natural history of the disease. Indeed, clonal evolution was observed in 10-20% of the patients who developed del 17p13, del 6q21, del 11q23, trisomy 8q24 at follow up studies Importantly, the late appearance of 11q- in CLL was associated with disease evolution.Thus, a modern diagnostic workup in CLL should include cytogenetic and molecular cytogenetic investigations, which should be performed before first line treatment and at relapse for the selection of risk-adapted treatment.

FLOW CYTOMETRIC ANALYSIS OF ZAP70: A MULTI-CENTER STANDARDIZATION STUDY

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The Flow Cytometric analysis of ZAP70 expression in chronic B lymphocytic leukemia (B-CLL) is of great importance, but it is hampered by the very low antigen expression and by the technical requirements of intracellular staining. The direct comparison of a single staining for ZAP70 with its isotype control is also charged with a great deal of intrinsic variability, that generates problems in the judgement of a positive or negative ZAP70 status.

The Italian Society for Cytometry – GIC has launched a multicenter study aimed at the evaluation of the analytical reproducibility of ZAP70 analysis, with a paired comparison between the In House method vs two different standardized protocols with centrally distributed reagents from BDB (Alexa-488) or IL-Coulter (PE). Ten centers participated to the BDB arm, ten to the IL-Coulter arm and six to both arms. Each center studied at least 5 untreated B-CLL patients at various clinical stages, with an extended leukemic phenotype and IgVH mutation status. A triplicate staining vs isotype control analysis was requested, with a matrix cross-comparison of the 9 possible results, using the % positive cell count and the ratioing criterion between ZAP70 and control intensities.

The preliminary data analysis showed a wide variability of the results, both with the In House and the standardized protocols, indicating the poor reliability of the % positive cell criterion. The need for stronger conjugates and a reliable fixing/permeabilizing system is underscored.

TUMOR IMMUNOTHERAPY: RESULTS AND PERSPECTIVES

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Several studies of the last few years indicate that a great progress has been made in basic immunology and the efforts have been made to translate such an information in the clinics for improving the prognosis of patients whose cancer is resistant to standard treatments like chemotherapy, radiotherapy and/or surgery and even to new molecular targeting agents.

Two main strategies may be considered in cancer immunotherapy: *adoptive immunotherapy* with immune cells, antibodies or other molecules that can be obtained thanks to the available molecular biotechnology, and the *active immunotherapy* or therapeutic vaccination.

Adoptive immunotherapy has been performed in the past, mainly in metastatic melanoma, using patient T lymphocytes obtained from the peripheral blood or from tumor infiltrating lymphocytes (TIL). These effector cells were expanded *in vitro* either with IL-2 only (LAK cells) or after specific activation with protein, peptide or autologous tumor cells and administered with high dose IL-2. In a recent variant of this strategy, tumor-specific lymphocytes were expanded *in vitro* with IL-2 and/or other cytokines (e.g. IL-7, IL-15) to further improve their therapeutic activity.

The early approaches of adoptive immunotherapy with LAK cells resulted in clinical tumor response (PR, CR, usually short lived) in approximately 30% of patients with metastatic melanoma though relevant toxic side effects occurred due to the high dose of IL-2 administered; moreover, the cost of this treatment was hardly affordable on large scale. After several years of limited work with this strategy, the group of Steve Rosenberg (NCI, Bethesda) has rekindled the adoptive immunotherapy of cancer by exploit-

ing the new information on the biology of T cells acquired by basic research studies that allows now a better selection of such cells in terms of antigenic specificity (even by transducing TCR genes) and duration of their survival and tumor cytotoxic function in treated patients. These last studies, though still accompanied with high toxicity caused by IL-2 administered together with lymphocytes and by the need to immune deplete the host with cytotoxic drugs or even total body irradiation, resulted in PR or CR in 70% of patients with metastatic melanoma, a remarkable clinical outcome.

As for *vaccination*, a strong development of this strategy occurred after the cloning (1991) of the first gene encoding a T cell specific melanoma antigen (MAGE). During the last 10 years several clinical studies have been performed with cancer vaccines based on, a) peptide expressed by human tumors (melanoma; prostate/lung/pancreas carcinomas, etc.); b) tumor cells genetically modified or on dendritic cells pulsed with protein antigen; c) recombinant vectors containing DNA or RNA sequences coding for antigens of different tumors. While a great deal of new immunobiological information has been collected through these translational studies, the tumor clinical response only rarely exceeded 20% while vaccine or tumor-specific T cell responses ultimately varied from 40 to 80%. However, the increased knowledge of molecular mechanisms of immunological escape by neoplastic cells and of the immune paralyzing tumor microenvironment, suggest that, by counteracting these immune inhibitory circuits, anti-tumor vaccines can become an effective therapeutic weapon in the near future even in association with immune-modulatory antibodies.

In fact, examples of clinical studies of vaccination with remarkable therapeutic efficacy have been recently presented in metastatic melanoma patients a) treated with the immune-modulating antibody to CTLA4, b) vaccinated in a prospective phase III randomized trial with gp100 peptide-based vaccine and IL-2, and c) in prostate cancer patients receiving a dendritic cell-based vaccine in a phase III study (Dendreon).

THE STEM CELL HYPOTHESIS IN SOLID TUMOUR

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Cancer stem cells (CSCs) are tumour cells which have stem features such us self-renewal, high migration capacity, drug resistance, high proliferation ability. In the last 15 years the pathological meaning and the existence of CSCs have been matter of discussion and a large number of articles have been published about the role that this cells play in the development and maintenance of tumours. It has been demonstrated in experimental models of human tumours that tumour lesions are built up by heterogeneous population of cancer cells and the presence of stem antigens can be evidenced by phenotypical analysis. Cytofluorimetric analysis can play an important role in this phase of identifying and recognize cancer stem cells. The most used markers have been CD34, but also CD133 and CD24, as markers of not-differentiated cells, often coupled with migration molecules as CD44, CD29, CD31 and other integrins. The different cell surface phenotypes prospectively identify tumour-initiating sub-populations in solid tumours and even cell lines derived from tumours retain hierarchical stem cell patterns demonstrable as differing clonogenic abilities related to cellular properties such as size, adhesiveness, dye exclusion, and patterns of gene expression. Actually it is not yet clear if the cancer cells with stem properties isolated within the tumours are born as mutated stem cells with tumouringenic activity or if the latter is the result of the recruiting of stem cells by the cancer, reprogramming the stem cell fate through factors released by cancer cells. Several hypotheses exist about the pathological activation of a stem cell. One hypothesis considers the signals that can reach the stem cells within their niches. A pathological condition can change the balance between the signals that come from the niche and the ones that come from surrounding environment, generating a signal that makes the cells exit out the niche without a clear differentiation fate, helping the formation of highly proliferative population. Oct-4, Wnt, Notch, Shh are genes involved in self-renewal and determination of differentiation fate. A deregulation of these signals can be often related with development and progression of a cancer stem cell. This experiments will be the second part of our study. In our preliminary data obtained by flow analysis testing solid tumors such as breast cancer, lung cancer, malignant melanoma and squamous cell carcinoma to test the presence of antigens to identify and then characterize cancer stem cells.

CIRCULATING TUMOR CELLS: BIOLOGY AND CLINICAL APPLICATIONS IN ONCOLOGY

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Circulating tumor cells (CTCs) often represent the first step of the metastatic process and their quantitative and functional study is leading to different applications in oncology practice. Over the past few years different separation procedures (most of them based on immunomagnetic capture) made possible an accurate enumeration of CTCs at extremely low frequencies in peripheral blood samples obtained from pts with advanced cancers. CTC count is already being used in several Oncology Departments and it provides informations that are clinically relevant.

When a quantitative approach is utilized, the baseline CTC count is an important prognostic factor within specific subgroups defined by treatment or patient characteristics. CTCs can also be utilized as a biologic surrogate marker of treatment response, becoming a tool for real-time assessment of the tumor outcome. Measuring CTCs before and after treatment gives an indication of whether or not the patient is responding, and trials are underway in breast, colorectal and prostate cancer, to verify the correlation with survival.

Moreover, in the *tailored cancer therapy era*, in which the treatment of several tumors is driven by a molecular

target, CTCs offer a "liquid biopsy" that make possible to characterize the tumor genotype (e.g. K-RAS mutations in colorectal cancer) during treatment with targeted therapies and then predict and monitor the clinical and molecular response of the tumor, by means of a non invasive approach.

In the field of translational research, the study of CTCs will permit to go deeper inside in a new integrated tumorigenesis model that involves different interdependent stem cells compartments, such as cancer stem cells, endothelial progenitors and mesenchymal stem cells. This will be possible by the development of sophisticated methodological and technical approaches to this new "rare event analysis", that will deserve clinical validation in the next future.

NK CELLS IN INNATE AND ADAPTIVE IMMUNITY

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In humans, two major NK cell subsets exist that display remarkable functional differences in their cytolytic activity, cytokine production and homing capabilities. In particular, CD56high CD16- NK cells that largely predominate in lymph nodes, have little cytolytic activity but release high levels of cytokines whereas CD56low CD16+ NK cells, that predominate in peripheral blood and inflamed tissues, display lower cytokine production but potent cytotoxicity. Various cell types that are resident within peripheral tissues as well as circulating cells (including NK cells), that have been recruited in response to chemokine gradients into inflamed sites, are equipped with receptors for pathogen-associated products that induce cytokine release upon engagement by their specific ligands. These cytokines directly influence the ability of NK cells to modulate both innate and adaptive immune responses. For example, innate cytokines such as IL-12 and IL-18, produced by antigen presenting cells (APC) including monocyte-derived dendritic cells (DC), by acting on NK cells at early stages of immune response, promote two distinct pathways of T cell priming each characterized by a sharp polarization towards Th1 priming. On the contrary, exposure of NK cells to an IL-4 rich milieu, resulting from the release of this cytokine by other innate immune cells such as mastocytes and eosinophils, leads to a deviation from Th1 responses towards non-polarized T cell priming. The polarizing effects of NK cells is exerted at two different stages: the first, taking place in peripheral inflamed tissues, is based on the "editing" process by which optimal maturation of DC is achieved, while the second is taking place in secondary lymphoid tissues where NK cells upon release of IFN-gamma directly influence T cell polarization towards Th1 responses.

ISOLATION AND CHARACTERIZATION OF T CELLS WITH REGULATORY FUNCTIONS

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Over the last 10 years thanks to the great amount of work performed in the field of T cell tolerance $CD4^+$ T cells with regulatory functions have been characterized. These cells have been shown to express the CD25 at high intensity and the Foxp3 gene. The constant use of more sensitive techniques able to isolate these cells have allowed the detailed characterization at cellular and molecular level of these cells. While on the one side it has been possible to understand the basic mechanisms by which these cells function it is also crucial to underline some of the aspects related to their isolation techniques on the other: regulatory T cell plasticity and how it can be maintained over time; the possibility to isolate cell populations with the highest level of purity but at the same time in enough numbers to allow clinical settings; isolation of novel Treg cell markers; the possibility to isolate "untouched" Treg cell populations; the possibility to characterize molecular and cellular alterations induced by the isolation procedure. These aspects will be critically analyzed and "the status of art" of the field will be provided.

CYTOMETRIC DIAGNOSIS OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)

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It is well accepted that flow cytometry enables detection and precise measurement of PNH clones. Nevertheless, the difficulty in identifying and quantifying PNH clones, even in expert hands, is low in classical PNH, high in PNH associated with other bone marrow disorder and very high in subclinical PNH. In spite of the enormous advancement in basic and clinical knowledge of PNH, diagnosis in clinical laboratories is still hampered by several difficulties leading to erroneous classification of patients. Major causes of diagnostic misclassification in PNH are i) low performance of monoclonal antibodies; ii) low sensitivity of flow cytometers; iii) low experience of operators; iv) not adequate knowledge of PNH subpopulation structure.

It is conceivable that PNH is still a sub-evaluated disease, and that a number of patients with PNH are misdiagnosed as hemolytic anemia, aplastic anemia or other syndromes.

We carried out a project (termed 'SCAPE') in order to improve the awareness of flow cytometrists of the difficulties in PNH diagnosis, ameliorate PNH diagnostic techniques, as well as stimulate critical attitude to evaluate reagents and instruments. To do this, a PNH diagnostic community (60 flow cytometrists) was created, based upon the exchange of PNH flow cytometry files. The files contained the following antibody combinations: 1. CD66b/CD33/CD45; 2. CD14/CD33/CD45; 3. FLAER/CD33/CD45; 4. CD59. Data of 20 patients with PNH were collected and shared with participant centres. Participating centres analyzed files and answered a series of multiple choice ques-

tions. The project lasted 12 months, including enrolment, file exchange, analysis of data and final meeting. A series of interesting results were obtained, since (i) specific flow cytometric techniques were largely diffused, (ii) the diagnostic panel was optimized with the introduction of FLAER, (iii) inter-laboratory precision was evaluated.

NOVEL CYTOMETRIC PLATFORMS FOR EUKARYOTIC FUNCTIONAL GENOMICS

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Eukaryotic organisms comprise complex mixtures of cells which coordinate gene expression between themselves in a way that efficiently regulates development and response of the organism to its environment. Elucidating these patterns of regulation, and the functions of the underlying and responding genes, loosely termed functional genomics, has been greatly facilitated by the recent development of high-throughput cytometric platforms for characterization of gene expression. Drawing from examples in my laboratory, in this talk, I describe three of these platforms, the way that they have been implemented and the results obtained.

The first explores global analysis of cell type-specific gene expression, and employs expression of the Green Fluorescent Protein within cells and within nuclei as a marker for selective purification, respectively, of cells and nuclei through fluorescence-activated sorting. These are then used as sources of polyadenylated RNA for microarray-based global gene expression profiling.

The second extends global analysis of cell type-specific gene expression to messages that are being actively translated on polyribosomes. This involves expression of epitope-tagged ribosomal proteins under the control of cell-type specific promoters. Epitope-tagged polyribosomes are then immunopurified from total polyribosomal preparations, and employed as the source of targets for microarray-based characterization of global transcript abundance.

The third allows characterization of the influence of genotype on global gene expression within plant populations, focusing on the important crop, rice. It is based on the identification *in silico* of Insertion-Deletion elements, which are then employed for design of microarray elements. These microarrays provide a highly cost-effective means for determining the genotypes of Recombinant Inbred Lines as well as their parents. Combining genotyping with expression profiling then permits elucidation of regulatory mechanisms as well as assignment of candidate genes to QTLs.

The future prospects of these cytometric platforms and others, either in development or envisaged, will be discussed.

References

Birnbaum K, Shasha DE, Wang JY, Jung JW, Lambert GM, Galbraith DW, Benfey PN (2003). A gene expression map of the *Arabidopsis* root. *Science* 302:1956-1960.

Zanetti ME, Chang I-F, Gong FC, Galbraith DW, Bailey-Serres J (2005). Immunopurification of polyribosomal complexes of arabidopsis for global analysis of gene expression. *Plant Physiology* 138:624-635.

Birnbaum K, Jung JW, Wang JY, Lambert GM, Hirst JA, Galbraith DW, Benfey PN (2005). Cell-type specific expression profiling in plants using fluorescent reporter lines, protoplasting, and cell sorting. *Nature Methods* 2:1-5.

Zhang CQ, Gong FC, Lambert GM, Galbraith DW (2005). Cell type-specific characterization of nuclear DNA contents within complex tissues and organs. *Plant Methods* 2005, 1:7 doi:10.1186/1746-4811-1-7.

Zhang CQ, Barthelson RA, Lambert GM, Galbraith DW (2008). Characterization of cell-specific gene expression through fluorescence-activated sorting of nuclei. *Plant Physiology* 147:30-40.

Edwards JD, Sweeney M, Janda J, Gaikwad A, Liu B, Leung H, Galbraith DW (2008). Development of a high-throughput, low-cost genotyping platform based on oligonucleotide microarrays, and its evaluation in rice. *Plant Methods* 2008, 4:13.

THE CYTOMETRY ACCREDITATION

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There is an increasing attention for professional accreditation in cytometry, in particular in flow cytometry. In fact, the need to assure quality and to control the clinical risk in laboratory test results, becomes more important for national requirements, also because the European Community aims to create a homogeneous healthcare level through several directives.

Specific requirements for institutional accreditation are not yet established in Italy. An important first step is the ISO certification, which may also facilitate the implementation of following specific accreditation, as it happens for the EFI accreditation in many HLA laboratories. A determinant role is played by the Italian Scientific Society (GIC), which can design and promote a shared model of professional accreditation, in order to implement it. The primary objective is to define criteria that make the quality and clinical safety continually adequate to the state of art, targeting the patient benefits. Therefore, the professional accreditation and related "peer review" are to be seen as an educational course, and not as an evaluation of the laboratory and operator performances: the aim is to promote a continuous improvement to excellence.

IMPACT OF DONOR NK CELL ALLORECOGNITION OF MISSING SELF ON HAPLOIDENTICAL HEMATOPOIETIC TRANSPLANTATION

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Natural killer cells influence HLA haplotype-mismatched hematopoietic transplants in a favourable way. NK cells possess inhibitory Killer cell Immunoglobulin-like Receptors (KIRs), that recognize groups of HLA-B and HLA-C class I molecules ("KIR ligands"). In KIR ligand-mismatched transplants, donor NK cells that express as their sole inhibitory receptor for self, a KIR for the HLA class I group which is absent in the recipient, sense the missing expression of the self class I ligand on allogeneic targets and mediate alloreactions. Donor NK cell alloreactivity reduces the risk of leukemia relapse, does not cause GvHD, and improves survival (Ruggeri et al., Blood 1999; Science 2002; Blood 2007; Stern et al., Blood 2008). In an updated analysis, 112 high-risk AML patients received haploidentical transplants from NK alloreactive (n=51) or non-NK alloreactive donors (n=61). Transplantation from NK-alloreactive donors was associated with a lower relapse rate and better event-free survival (EFS) in patients transplanted in any complete remission (relapse: 3% vs 47%, P<0.003; EFS: 67% vs 18%, P=0.02); overall reduced risk of relapse or death (relative risk vs non-NK-alloreactive donor: 0.48 [95% CI 0.29-0.78], P<0.001). More recently, we have documented that NK cell alloreactivity provided much better protection from leukemia relapse when exerted by maternal donors (as opposed to any other donor-recipient family relationship) (Stern et al., Blood 2008). The effect was independent of, and additive to, the beneficial effects of NK alloreactivity, suggesting that exposure to fetal antigens during pregnancy resulted in long-lasting memory T cell immunity against the child's paternal HLA haplotype.

The mechanism whereby alloreactive NK cells exert their benefits has been elucidated in mouse models. Pretransplant infusion of alloreactive NK cells ablates 1) leukemic cells, 2) recipient T cells which reject the graft, and 3) recipient dendritic cells (DCs) which trigger GvHD.

BONE MARROW MICROENVIRONMENT AS A THERAPEUTIC TARGET IN HEMATOLOGICAL MALIGNANCIES: THE MYELOMA LESSON

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In the last years several evidences indicate that microenvironment have a critical role in the pathophysiology of hematological malignancies. Particularly, Multiple myeloma (MM) is is characterized by the tight relationship with the bone marrow (BM) microenvironment that has a pivotal role in the regulation of myeloma cell growth and survival through the production of several growth factors and the increase of BM angiogenesis. Bone microenvironment cells as osteoclasts and osteoblasts also support the survival of MM cells. The study of the alterations of the microenvironment and that of the interactions between MM and microenviroment cells has leaded to identify new therapeutics targets in MM patients and to develop new drugs targeting both MM cells and the microenvironment including endothelial cells, T lymphocytes osteoclast and osteoblast. Recently authors have studied the alterations occurring in the bone cells in MM patients, the biological mechanisms involved in the interactions between MM cells and osteoprogenitor/osteoblastic cells and the potential role of osteoblastic cells in the anti-tumoral effect of the new drugs. In the last years several new anti myeloma drugs mainly targeting the bone marrow microenvironment as proteosome inhibitors Bortezomib e Carfizomib, Thalidomide, Lenalidomide and Pomalidomide have been introduced in clinical trial and practice in multiple myeloma patients. Moreover growing evidences suggest that these drugs may affect osteblast and osteoclasts even if their effects on multiple myeloma bone disease are not completely known.

CELL CYCLE AND APOPTOSIS

AN ULTRASTRUCTURAL APPROACH TO THE STUDY OF APOPTOTIC DOUBLE STRAND DNA CLEAVAGE

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Apoptotic DNA cleavage initially produces large fragments (50 kbp), followed by the formation of nucleosomic/oligonucleosomic ones. On the other hand, apoptosis without DNA fragmentation, at least the nucleosomic one, has been described. To study the correlation between the DNA cleavage and the well known chromatin behavior, we applied TUNEL to electron microscopy, by using a gold-conjugated antidigoxigenin antibody, on U937 and Molt-4 cells, both exposed to UVB or staurosporine. Gold particle density in the different domains of apoptotic nuclei was statistically evaluated. Gold labelling was more intense in dense apoptotic chromatin than in the diffuse one. U937 cells, which evidenced in vitro oligonucleosomic fragmentation after both treatments, revealed a significantly higher gold particle density, when compared with Molt-4, which did not, even if showing larger DNA fragments in vitro. DNA fragment sizes, characterized by gel electrophoresis and by FIGE, appeared closely correlated to gold particle density on apoptotic chromatin domains. TUNEL applied to electron microscopy is an useful tool to highlight mechanisms underlying apoptotic chromatin condensation and DNA cleavage patterns.

INVOLVEMENT OF THE HISTONE ACETYLTRANSFERASE P300 IN NUCLEOTIDE EXCISION REPAIR

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Exposure to genotoxic agents may induce the formation of a variety of DNA lesions that cells must remove in order to avoid genomic instability, and to prevent cancer formation. To this end, cells have developed genome surveillance and signalling pathways (checkpoints) that are flanked by DNA repair systems. Recent findings have shown that cell cycle checkpoints and DNA repair systems cross-talk each other. However, the role and the molecular mechanisms underlying these connections are not yet well understood. The cell cycle inhibitor $p21^{CDKN1A}$ is directly involved in nucleotide excision repair (NER), thanks to its interaction with PCNA, and with the histone acetyltransferase (HAT) p300. Previous studies suggested that p300 HAT activity might be required in NER, by providing chromatin accessibility to DNA repair factors. In addtion, it was shown that p300 interacts with PCNA, and that p21 regulates p300 HAT activity by dissociating interaction with PCNA. Since p300 may also associate with other NER factors, such as XPA, or DDB2, it has been suggested that p300 may play an additional role by regulating directly some step of the repair process. To further understand the involvement of p300 in DNA repair, we have investigated the influence of p300 knock-down on NER efficiency by RNA interference (RNAi). We have assessed NER activity after treatment of primary human fibroblasts with siRNA to silence p300, and/or its homolog CBP, in order to analyze the specificity of the process. NER activity has been evaluated by analyzing unscheduled DNA synthesis (UDS) activity through autoradiography of ^{3}H-thimidine incorporation, after UV irradiation. Concomitantly, cell viability has been determined with the MTT test. The results have indicated that p300, but not CBP silencing, reduces significantly the number of autoradiographic grains. In addition, cells treated with siRNA to p300 have shown a reduced viability after UV irradiation, as compared with cells treated with control siRNA molecules. In order to identify a particular step of NER requiring p300 activity, we have analysed the recruitment of PCNA at locally-damage sites, after p300 silencing. No significant difference in the recruitment of PCNA, has been found in p300-siRNA treated cells, suggesting that p300 activity is required at a down stream step, thereby impairing UDS activity. (Work supported by AIRC).

APOPTOSIS INDUCED BY VERBENA OFFICINALIS OIL AND CITRAL IN PERIPHERAL BLOOD LYMPHOCYTES FROM NORMAL AND CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) PATIENTS

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Preliminary evidence indicates that isoprenoids, a broad class of mevalonate-derived phytochemicals which are ubiquitous in the plant kingdom, may act, with great potency, by inhibiting the tumor cells proliferation of human breast adenocarcinoma (MCF7), human leukemia (HL-60) and human colon adenocarcinoma (CaCo 2). *Verbena Officinalis L.* (Verbenaceae), commonly known as vervain, is a medicinal plant whose essential oil is mainly constituted by isoprenoids. The plant and/or its essential oil have been widely used in different traditional medicines. The plant was approved as a herbal medicine and dietary supplement by several regulatory acts of many Countries. Despite its widespread use, the mechanisms of pharmacological actions of the herb or the volatile oil are still unclear. We have screened 13 CLL patients at onset, for some typical chromosomal rearrangements, namely, trisomy 12, del 13q14, rearrangements of 14q32, 11q22.3 (ATM), and 17p13 (p53).The aim of the study was to verify the ability of undergoing apoptosis in the presence of the isoprenoid compounds deriving from Verbena Officinalis (Vervain) and their main component Citral, that have been previously reported as showing anti-cancer properties. Peripheral blood lymphocytes from healty donors with normal karyotype, were tested in the same experimental conditions. Vervain oil induced apoptosis both in normal and CLL cells after short term (4, 8, 24 hrs) culture. The percentage of apoptotic cells was significantly (Student's T test) higher in CLL patients. The presence of del 17p13, was associated to a reduced ability to undergo apoptosis compared with other rearrangements or nomal karyotype. Our data agree with the literature in indicating natural compounds as possible precursors of new therapeutic agents.

ISOTHIAZOLINONES-INDUCED APOPTOSIS IS ASSOCIATED WITH ALTERATION OF INTRACELLULAR CALCIUM HOMEOSTASIS

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Previously we reported that brief exposure of HL60 cells to a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one (CMI) and 2-methyl-4-isothiazolin-3-one (MI) shifts the cells into a state of oxidative stress that induces apoptosis and necrosis.

In this study we investigated the role of calcium and of glutathione level modulation in the molecular mechanism of apoptosis and necrosis induced by CMI/MI in HL60 cells. We performed the analyses using flow cytometry: cytoplamic calcium levels, mitochondrial calcium levels, changes in mitochondrial transmembrane potential (Dym), DNA content were assessed by FuraRed-AM, Rhod-2, Rhodamine 123, propidium iodide and RNAse, respectively. Here, we show that CMI/MI induces early perturbation of calcium homeostasis, increasing cytosolic and mitochondrial calcium and depleting the intracellular endoplasmic reticulum stores. The sustained increase in Ca^{2+} followed by mitochondrial Ca^{2+} uptake causes

mitochondrial hyperpolarization. Pre-incubation of cells with GSH-OEt, which enhances cellular GSH content, reduced cytosolic and mitochondrial calcium levels, thus protecting against mitochondrial dysfunction, apoptosis and necrosis shifting to apoptosis.

Our results suggest that different intracellular calcium pools are involved in CMI/MI cytotoxicity and that the degree of GSH depletion, may have a causal role in increasing calcium levels. Hence we propose that in an early phase of its action, CMI/MI induces a calcium release from ER stores followed by mitochondrial calcium loading, which in turn triggers mitochondrial impairment and cell death.

STUDY OF CELL CYCLE MODIFICATIONS IN AN IN VITRO MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Background: Neuroblastoma cell line SH-SY5Y transfected with the mutant SOD1 (G93A) gene is a well-known AmYotrophic Lateral Sclerosis (ALS) cellular model. Alterations of regulators of G1 to S cell-cycle phase like cyclin D1, CDK4, ppRb and E2F-1 have been previously described in ALS. p27 is a Cip/Kip family member inhibiting progression from G1 to S. Stathmin (Op18) has a key role in cell cycle progression and its expression was altered in G93A transgenic mice. ROS are thought to be toxic, but there are evidences that they have a role in cell cycle.

The aim of this study was to investigate the possible role of SOD1 in cell cycle. Expression of cell cycle regulators p27 and Op18, activity of Op18 evaluated by phosphorylation on Ser16, cell cycle position and ROS levels have been studied in SH-SY5Y cell line, transfected with wild-type (WT) or mutant SOD1 (G93A).

Methods: ROS levels and alterations of cell cycle progression were assessed by flow cytometry; cells were synchronizated with a serum-deprivation protocol to highlight differences between the cell lines. Protein expression was evaluated by Western Blotting. Op18 phosphorylation was studied with In-Cell Western Assay.

Results: After synchronization, cells were significant in G1 phase. Flow cytometry analysis after 20 hours showed higher distribution of WT cells in G1 phase, whereas G93A were more in S and G2/M phases ($p<0.05$). There were no differences in Op18 expression levels. p27 expression was higher in WT than in G93A and Op18 was more phosphorylated in G93A than WT ($p<0.05$), in agreement with cell cycle results. Growth curve was consistent with these results. There were no significantly differences in ROS levels.

Discussion: These data suggest that overexpression of WT or mutant SOD1 can alter cell cycle progression. Since there were no differences in ROS levels and any direct interaction between SOD1 and stathmin or p27, experiments are in progress to evaluate the involvement of the regulators Bcl-2 and CdK4.

ANALYSIS OF OXIDATIVELY MODIFIED WT SOD1 IN PATIENTS' LYMPHOBLASTS (ALS) BY CONFOCAL MICROSCOPY AND FLOW CYTOMETRY

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Background. Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disease characterized by the selective loss of motor neurons in the brain and spinal cord. 90% of ALS cases are sporadic (SALS). About 10% of cases are familial (FALS) and approximately 25% of FALS patients inherit autosomal dominant mutations in the gene encoding copper-zinc superoxide dismutase (SOD1). Recent studies suggest that Wild-Type SOD1 (WTSOD1) may acquire aberrant and toxic properties similar of those of the mutated SOD1 in at least a subset of SALS, being a major target of oxidative damage.

Objectives. Our hypothesis is that due to age and/or environmental associated oxidative damage, WTSOD1 undergoes post-translational and/or conformational modifications similar to those caused by SOD1 mutations. This study is aimed at identifying by flow cytometry and confocal techniques differences in SOD1 expression and/or conformation between patients and controls.

Methods. Lymphocytes derived from 3 FALS, 6 SALS and 5 healthy controls were immortalized using EBV virus and treated with 100μM hydrogen peroxide. Confocal analysis and flow cytometry: Cells were plated on poly-L-lysine pre-coated slides or simply collected and then fixed using 4% paraformaldehyde. Samples were treated with a blocking solution incubated o/n at 4°C with anti-SOD1 antibody and then with secondary antibody.

Results. Using patients' lymphoblasts we were able to show disease specific properties of WTSOD1.

Confocal and flow cytometry analysis, at basal level and after H2O2 treatment, shows an higher fluorescence of WTSOD1 in FALS and SALS lymphoblast. Since no differences in protein expression have been found analyzing lymphoblasts' total lysate by Western Blotting, we suppose that this discrepancy may be due to a protein conformational change attributable to an altered pro-oxidant cell environment.

Discussion and conclusion. These results suggest that at least in a subset of sporadic patients WTSOD1 may acquire binding and toxic properties similar to those observed in mutant SOD1. Studies are underway to test whether these modifications are indeed toxic and participate in the pathogenic mechanism(s) underlying the disease in SALS and to verify if cytometry could be used as an efficient diagnostic technique of the pathology.

ENVIRONMENTAL SCIENCES AND TOXICOLOGY

THE EFFECT OF POLYUNSATURATED ALDEHYDES ON MARINE PHYTOPLANKTON AND BACTERIA

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Some diatoms are known to produce polyunsaturated aldehydes (PUAs) upon disruption of their cell membrane by grazing or cell lysis. Bacteria live in strict contact with diatoms at sea and are therefore presumably exposed to the chemicals they release. However, the nature of this interaction and the role of PUAs in it still remain poorly understood. We tested the effects of three PUAs and a mix of two on two different natural communities of bacteria from the Mediterranean Sea. One site, along the northern Spanish coast in the Catalan Sea, was sampled in May 2007. The other, along the Italian coast of the Northern Adriatic Sea, was sampled during a diatom bloom in February 2008. In both cases, no or little effect on cell concentration or total bacterial production was observed when compared to the control. Only slight modifications of community composition (CARD-FISH) were observed, while metabolic activity (Mar-CARD-FISH) indicated a group-specific reaction to the PUAs inoculated, with Roseobacter the most affected and Gammaproteobacteria the least. Hence, PUAs appear to determine community composition directly by favouring some bacterial group, or indirectly, by slowing down some others. This has implications for the diversity of bacterial communities when diatoms are present, and suggest specific interactions between certain groups of bacteria and diatoms. Although we cannot imply an antimicrobial effect of PUAs, there is evidence that some bacteria use their resistance to PUAs as a tool to outcompete competitors for the same resources.

WINTER DISTRIBUTION AND DYNAMICS OF PICOPLANKTON IN THE NORTHERN ADRIATIC SEA

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In the northern Adriatic Sea picoplankton (autotrophic and heterotrophic) distribution and dynamics were investigated during a cruise in February 2008. During the cruise 32 stations were sampled at surface, bottom and 1 to 7 intermediate depths. Cell concentrations were estimated by flow cytometry for two main groups of autotrophs (*Synechococcus* spp, and picoeukaryotes, the latter represented by several clusters in the cytograms), and the heterotrophic bacteria. Vertical distribution was generally homogeneous, as consequence of strong seasonal mixing of the water column, except at the coastal station close to the Istria peninsula, probably due to local haline stratification.

Synechococcus spp. represented the most abundant group of autotrophic picoplankton, with an average of 3.90 10^4 cell ml^{-1} (SD 3.36 10^4 cell ml^{-1}), while picoeukaryotes were on average 2.35 10^3 cell ml^{-1} (SD 2.35 10^3 cell ml^{-1}) and heterotrophic bacteria 7.14 10^5 cell ml^{-1} (SD 2.22 10^5 cell ml^{-1}).

At surface, *Synechococcus* spp. showed higher concentrations in the northern part of the sampled area with a peak close to the Croatian town Rovinj, while picoeukaryotes were mainly located along the Italian coasts, so as heterotrophic bacteria.

Total C of the pico-fraction at surface, estimated from conversion factors, was on average 31.91 μg C l^{-1} (SD 16.39), 16.70 μg C l^{-1} (SD 11.80 μg C l^{-1}) of which attributable to the autotrophs. The latter represented from 15 to 86 % of phototrophic C estimated from chlorophyll on 19 stations. Within the picoplankton, the autotrophs represented 52% of total picoplankton C.

Growth and grazing rates of autotrophs were also estimated from dilution experiments at two coastal stations, V7, located in proximity of the Po river delta and V13, in the coastal area in front of the town of Cesenatico. Estimated growth and grazing rates were lower when compared to previous data, and showed high variability.

MECHANISMS OF RADIATION EFFECTS AT CELLULAR LEVEL: BYSTANDER EFFECTS AND MODULATION OF THE CYTOKINE SIGNALLING

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A basic paradigm in radiobiology is that, after exposure to ionising radiation, the deposition of energy in the cell nucleus and the resulting damage to DNA are responsible for the harmful biological effects of radiation. However, over recent years it has been shown that the mechanisms of action of radiation include the initiation of cascade of events referred to as non-targeted effects. For example, cells neighbouring those which have been irradiated respond to signals released from irradiated cells (i.e. bystander effects). Evidences accumulated so far suggest that these effects are mediated by the diffusion of one or more factors, among which cytokines are likely to play a key role. The studies available in the literature on bystander effects are still conflicting and extensive

research is needed aimed at understanding the mechanisms responsible of the effects observed in bystander cells.

The aim of this work has been the study of the release modulation of relevant cytokines (i.e. IL-6 and IL-8) and their respective receptor expression after *in vitro* gamma irradiation of human fibroblasts. In addition, to better investigate the signalling pathways involved, the effects of scavengers of the early-step bystander involved molecules, such as NO, ROS and OH radicals were investigated. Our evidences suggest that gamma rays exposure affects the production of both IL-6 and IL-8 and their receptor expression although with different features, suggesting different roles in the transmission of bystander effects. The observations collected with the presence of c-PTIO and DMSO in the culture medium suggest the existence of complex networks of regulatory mechanisms that modulate the bystander signals. Finally, these experimental results were used to implement mathematical models and simulation codes aiming to improve our knowledge on the basic mechanisms underlying intercellular communication.

SPATIAL AND TEMPORAL CHANGES IN PICOPLANKTON COMMUNITIES IN AN ITALIAN RIVER

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Rivers are important to society, providing waters to consumption, agriculture, recreations and for carrying away human wastes. On the other hand, surface waters may be strongly affected by anthropic activities. Picoplankton are very sensitive to water quality and play a crucial role in food webs and in the recycle of nutrients in aquatic environments.

The aim of this study was to evaluate changes in picoplankton communities along the Foglia River by FCM and CARD-FISH. Both autotrophic and heterotrophic components were analyzed and relationship with abiotic parameters was taken into account to better understand picoplankton distribution patterns linked to changing in chemical and physical water characteristics.

Changes in abundance, viability, activity and composition were registered and strong correlations with different environmental factors were observed, which underlined as with so many factors may control riverine picoplankton community abundance and composition.

Flow cytometric analyses revealed that bacteria populations tended to cluster into different fractions (High Nucleic Acid and Low Nucleic Acid cells), characterized by different fluorescence and scattering signals, with a general predominance of HNA cells. Bacteria community composition, evaluated by CARD-FISH, varied over the seasons and among the stations, with a high predominance of β-*proteobacteria*, followed by α-*proteobacteria*, *Cytophaga-Flavobacterium* and γ-*proteobacteria*.

In conclusion, this kind of studies on the analyses of picoplanktonic communities may help to better understand how ecosystems will respond to changes in environmental conditions.

EVALUATION OF TOXICANT INHIBITION IN WASTEWATER TREATMENT PLANTS COMPARING FLOW CYTOMETRY AND RESPIROMETRIC TESTS

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The effect of toxicants on bacteria of activated sludge taken from full-scale wastewater treatment plants were investigated comparing conventional and advanced promising methods. Plate counts (conventional approach), respirometric tests and flow cytometric analyses (advanced approaches) allowed to compare the replication inhibition, the respiratory activity inhibition and the esterases activity inhibition, respectively. The inhibitory effects originated by two reference toxicants (3,5-dichlorophenol -3,5-DCP- and $ZnSO_4 \bullet 7H_2O$) and a landfill leachate was investigated on activated sludge. Samples of landfill leachate were chosen because usually characterised by significant concentrations of metals and xenobiotics.

Flow cytometric assay was applied for evaluating the inhibition by these toxicants on both disaggregated and intact flocs, in order to discriminate:

- viable and dead cells, on the basis of membrane integrity/permeabilisation by coupling SYBR Green I and Propidium Iodide staining;
- enzymatically active cells, able to hydrolyse fluorogenic substrate such as BCECF-AM.

In this way, cellular damages caused by toxicants were investigated monitoring enzymatic activity reduction and/or membrane permeabilisation variations, as a function of toxicant dose and contact time. These results were compared with the decrease in the respirometric activity (measured by respirometric tests) and in the replication ability.

The obtained results proved that activated sludge was inhibited by 3,5-DCP and landfill leachate, depending on toxicant concentration and contact time, whereas it was less inhibited by $ZnSO_4 \bullet 7H_2O$; Zn is a common metal present in sewage treatment plants, which usually causes cell aggregation and flocculation of sludge flocs.

REAL-TIME DETECTION OF ACTIVE COLIFORMS AND ESCHERICHIA COLI IN WASTEWATER BY FLOW CYTOMETRY

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The aim of the research is the evaluation of a rapid procedure for the real-time and presumptive quantification of active coliforms and *E. coli* in wastewater samples, useful for monitoring the treatment and the disinfection processes. Two fluorogenic substrates, C_{12}FDG and C_{12}FDGlcU, were used to the detection of positive β-galactosidase bacteria (β-gal-positive cells considered as active coliforms), and positive β-glucuronidase bacteria (β-glu-positive cells considered as active *E. coli*), by single cell assay with FCM. This fluorogenic substrates contains a lipophilic group that allows the substrate to penetrate through cell membranes, to be hydrolyzed by intracellular β-gal and β-glu in a green fluorescent product, retained inside the cells. Enzyme activities are more persistent under environmental conditions than the culturability of target bacteria; therefore enzyme activities could be detected in wastewater and water samples both for culturable and active-but-not-culturable cells.

The proposed FCM approach – a multiparametric single-cell analysis performed using green fluorescence (related to enzymatic activity) and scattering signal (related to bacteria size and shape) – was applied to mixed bacterial populations in wastewater (29 wastewater samples). The FCM analysis is rapid, requiring 20 minutes to be completed and therefore suitable for real-time monitoring in wastewater treatment plants. The concentration of active coliforms and *E. coli* in wastewater measured by FCM resulted 3 orders of magnitude higher than the concentration of coliforms and *E. coli* measured by conventional cultivation methods, due to the enzymatically-active-but-non-culturable state of most cells after their discharge in the environment. Nevertheless the analysis presents some false-positive cases causing a small overestimation of true values, it is considered sufficiently accurate, being compatible with early warning applications.

DIFFERENT EFFECTS OF A 50HZ MAGNETIC FIELD ON HUMAN PERIPHERAL BLOOD CELLS

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The interest in the evaluation of health effects induced by extremely low frequency (ELF) electromagnetic field (EMF) exposures has largely increase in the last decades, mostly motivated by the occupational and environmental exposure of humans to such nonionizing fields.

Concerning in vitro studies, literature data are available on different cellular targets related to cancer risk, such as genotoxic effects, gene expression and cell proliferation. The exposure system was provided by Centro Sistemi Acustici Audiovisivi ed Elettromagnetici - C.S.A.A.E. (University of Urbino). Briefly, our systems include two cell incubators, one without any magnetic field, one with a solenoide able to create a 50 Hz ELF-EMF. The aim of this study was to investigate the effects of short (4 days) and long (10 days) term exposure on different cells by evaluating its influence on apoptosis, senescence and proliferation.

We tested leukocytes and U937 cells, both isolated and exposed to magnetic field, for apoptosis degree (by hypodiploid peak detection and supravital PI uptake), surface molecule (CD11a, 11b, 11c and CD54) expression and proliferation (by CFSE staining).

Our findings on leukemic U937are in agreement with data reported by Oda and Koike (2004), who found a slight decrease in spontaneous apoptosis, whereas on untreated whole blood leukocytes, stored for prolonged time, exposure to 50 Hz magnetic field seems to enhance apoptotic phenomena and to increase fluorescence intensity (FI) of CD11a and 11b, particularly on mono- and granulocytes. Furthermore preliminary data on CFSE staining suggest a high cell division rate for PHA-treated lymphocytes.

THE ECOLOGICAL ROLE OF MARINE DIATOM POLYUNSATURATED ALDEHYDES: EVIDENCES AND HYPOTHESES

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Polyunsaturated Aldehydes (PUAs) are produced by diatoms by a wound-activated mechanism and are responsible for egg hatching reduction in copepods, both in the laboratory and at sea. Apart from affecting survival and recruitment of their predators, PUAs have several other functions in the pelagic ecosystem, being also toxic for diatoms as they impair cell growth, causing cell death by an active mechanism closely resembling apoptosis. At relatively low concentrations, PUAs act as infochemicals within the diatom populations, triggering a stress signalling mechanism mediated by calcium and nitric oxide. PUAs also inhibit growth of other non-PUA-producing phytoplankton with different effect on different species. Even more so on marine bacteria, for which three different reactions have been evidenced: no effect, growth inhibition, growth stimulation. The assessment of these effects in situ is complicated by different factors interplaying. However, dissolved PUAs have been detected during diatom blooms, suggesting that there is release without grazing and that PUAs may have a similar effect as observed in culture, i. e. differential inhibition of organisms surrounding diatoms. Therefore, PUAs can have an important effect in determining plankton community composition during diatom blooms, and therefore biodiversity in general in terms of species present. As biodiversity is strictly linked to function, PUAs also influence ecosystem functioning, and this may have profound implications for the general role of the coastal areas where diatoms are abundant.

EVALUATION OF DIRECT-OXIDATIVE DNA DAMAGE IN LUNG AND BRONCHIAL HUMAN CELLS EXPOSED TO LOW DOSES OF HEXAVALENT CHROMIUM

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Cr (VI) compounds, are commonly used in several industrial processes and could represent an important widespread pollutant. Although the genotoxicity of Cr (VI) is well known and a role for oxidative DNA damage has been suggested, the mechanism of action is still not elucidated. We used Fpg-modified comet assay to assess direct-oxidative DNA damage on human lung epithelial (A549) and normal bronchial (BEAS-2B) cells exposed to 0.1, 0.5, 1.0 and 10μM sodium chromate for 0.5h, 1h and 4h. Our findings on A549 cells showed a time-dependent direct DNA damage, expressed as tail DNA%, beginning from 0.5 μM. For oxidative DNA damage an induction after 30 min to 0.5μM decreasing with time, and a time-dependent increase after exposure to 10μM was found. It indicates that at low Cr(VI) concentration the oxidative stress represents the first event followed by direct DNA damage while at the highest concentration a time-dependent increase of oxidative DNA damage was induced. On BEAS-2B cells a direct DNA damage induction was shown within 1h for 0.5, 1.0 and 10 μM exposure without changes with time showing the ability of this cellular type to resist to genotoxic effect of chromate unlike A549 cells. Moreover on BEAS-2B an early oxidative DNA damage at lowest concentration decreasing with time was found. The findings show an higher responsiveness of A549 cells to genotoxic effect of Cr(VI) demonstrated by time-dependent direct-oxidative DNA damage induction and an early transient oxidative DNA damage in Beas-2B. The results show the suitability of this experimental model, using the principal target cells of inhalation exposure and the sensitive comet assay to evaluate the different early genotoxic cellular response of normal and transformed cell to non-cytotoxic concentrations of metal compounds on target organ.

HIGH SURVIVAL OF FROZEN PBMCs IRRADIATED WITH GAMMA RADIATION

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Cell cryopreservation and their storage in liquid nitrogen offer the most secure method of cell preservation. However, cryopreserved cells are susceptible to ionizing radiation (IR) effects. A lot of experiments demonstrated that IR can induce cell death (mainly apoptosis), tumours and ageing in living cells, but only few information regarding the response of cryopreserved cells are available.

To investigate the effect of IR on frozen cells, peripheral blood mononuclear cells (PBMCs) obtained from freshly collected blood samples were isolated, frozen and then irradiated in liquid nitrogen. Frozen PBMCs were irradiated at relatively low doses (0.1, 0.3 and 0.9 Gy), intermediate doses (3.0 Gy) and high doses (18.6 Gy) of gamma rays (662 KeV del ^{137}Cs). After thawing and incubation (37°C, 5% CO_2) for 0, 24, 48, 72 and 96 hours, cell death has been evaluated by flow cytometry. Percentages of cell death induced by IR were revealed using both hypodiploid peak detection and supravital propidium iodide staining.

Below 0,3 Gy dose radiation, percentages of cell death were usually low and no significative apoptosis was detectable. Interestingly, low dose radiation hypersensitivity, typical of fresh cells, was not observable in frozen PBMCs. In fact, cell death gradually increase both with dose radiation and incubation time. We propose that both reduced free radical formation at low temperature and impaired functionality of apoptotic signalling after thawing might be responsible for increased survival and hypersensitivity loss at low dose radiation.

SPERM CHROMATIN INTEGRITY IN A SOUTH AFRICAN POPULATION LIVING IN A MALARIA AREA PERIODICALLY EXPOSED TO DDT

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The Stockholm Convention, a global agreement adopted in 2001 aiming at protecting human and environmental health from the effects of exposure to dangerous and ubiquitous chemical compounds, has banned a dozen of chemicals known as persistent organic pollutants (POPs), a family of man-made chemicals which includes polychlorinated biphenyls (PCBs), dioxins, furans, and also DDT (1,1,1-trichloro-2,2-bis(chlorodiphenyl)ethane). However, for the latter, exemptions are available for countries that are still using it to combat malaria. Several reports have indicated that DDT and its main metabolite p,p'-DDE, like other organochlorine pesticides, are not only toxic but may behave as endocrine disruptors and, as such, may impair wildlife and human fertility. There is mounting evidence that deteriorated semen quality can be associated with increased serum concentration of DDT and its metabolites in several populations worldwide. The problem is exacerbated in those situations where DDT is sprayed periodically to control anopheles mosquitoes where its plasma concentration can reach thousand-fold the level found in other geographically distant populations. However, there are limited and contradictory epidemiological data on whether DDT can also damage sperm DNA. Therefore, in order to investigate the possible adverse effects on human sperm genetic integrity in a sufficiently large heavily polluted population characterized by an adequate exposure contrast, we have set up a cross-sectional study involving 209 young males recruited in an endemic malaria area (Limpopo Province, South Africa) where DDT is sprayed annually. DDT and p,p'-DDE levels were measured in plasma. The flow cytometric sperm chromatin structure assay (SCSA) was used to assess sperm DNA/chromatin integrity. The lipid adjusted

p,p'-DDT (mean±SD) concentration was 109.2±106.6 μg/g lipid whereas the p,p'-DDE concentration was 246.2±218.5 μg/g lipid, among the highest blood levels measured so far in a reproductive toxicology human survey. A weak association emerged between DDT/DDE plasma concentration and the incidence of sperm with chromatin defects, suggesting that environmental DDT exposure might have a negative impact on the male gamete integrity of young South Africans.

TREATMENT OF STAPHYLOCOCCUS AUREUS BIOFILM BY A PHOTOCHEMICAL TECHNIQUE (PDT) COMBINED WITH ANTIBIOTIC OR HOST DEFENCE MECHANISM

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Staphylococcus aureus is one of the most important etiological agents of infections associated with medical devices. This is in part due to the ability of the organism to form biofilm, which provides a microenvironment that protects from attack by the host's immune system and by antibiotics.

In this study we examined the structure of polysaccharide intercellular adhesin (PIA)-dependent or protein-based *Staphylococcus aureus* biofilms.

We defined new strategies aimed at treatment of mature established biofilms using photodynamic treatment (PDT) combined with chemotherapy or phagocytosis.

Significant inactivation of bacteria was observed when structurally distinct biofilms were exposed to the cationic porphyrin, tetra-substituted N-methyl-pyridyl-porphine (TMP), and simultaneously to visible light. Moreover, PDT-treated biofilms exposed to vancomycin or subjected to the phagocytic action of whole blood resulted in their almost complete eradication. The drastic reduction in staphylococcal survival and the disruption of biofilms were confirmed by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM).

The results suggest that PDT combined with vancomycin and the host defences may be a useful approach for the inactivation of staphylococcal biofilms adhering to medical implant surfaces.

BYSTANDER EFFECT STUDIES IN HL60 HUMAN PROMYELOCYTES

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HL60 cell line is capable to differentiate *in vitro* into granulocyte-, monocyte- or macrophage-like cells. It is used in bystander effect studies aimed at investigating if micronuclei (MN), cell killing and cell differentiation can be induced by signals released after irradiation from the same cell type in various differentiating conditions.

Monocyte- and macrophage-like differentiation was obtained using vitamin D3 and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), respectively. Irradiation was performed with g-rays and bystander studies were carried out using the medium transfer approach.

The experiments were performed to obtain information about the response to signaling factors from sham/irradiated HL60 in terms of cluster of differentiation (CD) expression and time-modulation by Flow Cytometry bi-parametric analysis, MN induction and cell killing.

The CDs experiments have shown that CD11c CD13 and CD33 appear suitable for identifying macrophage-like and monocyte-like cells. However, the former are negative while the latter are positive to CD14. AP cells present a high positivity to CD13 and CD33, like macrophage-like cells but not to CD11c or CD14. This CDs will be used to distinguish promyelocytes from monocyte-like and/or macrophage-like cells in a mixed population in the bystander studies.

The results on MN induction in unirradiated AP cells by the medium collected from AP cells irradiated with 0.5 Gy and incubated for different times at 37°C have shown a significant effect after 2 h incubation that disappears at longer times. Cell killing data have shown that incubation with conditioned medium from both sham and 0.5 Gy irradiated AP cells leads to an increase in the plating efficiency of the bystander cells. However, this effect seems more related to factors physiologically released by the cells than to factors induced by irradiation. These results suggest that growth stimulating factors, instead of growth inhibiting factors, are released by HL60 promyelocytes. This work is supported by the EC NOTE Project (FP6-36465).

AN INTEGRATED PROCEDURE FOR THE ASSESSMENT OF VIABLE AND ACTIVE BACTERIA BIOMASS IN WASTEWATER AND ACTIVATED SLUDGE

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The most widely applied treatment of municipal wastewater is based on biological processes, such as activated sludge, in which the degradation of organic matter occurs by means of aerobic or anaerobic heterotrophic bacteria. The most common measurement for quantifying activated sludge in biological reactors is the content of Total Suspended Solids (TSS). In spite of many research works conducted on activated sludge and influent/effluent wastewater, no routinary methods are available nowadays to quantify rapidly the bacteria biomass in activated sludge and wastewater. The reason is related to: (1) non-culturability of most bacteria in activated sludge and wastewater, (2) a large presence of non-biotic particles which interfere with the microscopic observations, (3) the difficulty to disaggregate activated sludge flocs without losses in viability, (4) the

difficulty to convert the number of bacteria into an equivalent biomass.

In this research we propose an integrated approach based on flow cytometry (FCM) to solve some of the problems of bacteria biomass quantification in activated sludge and wastewater. It allows to overcome the limitations indicated above, and in particular:

– all bacteria are quantified by using fluorescent dyes able to stain nucleic acids, independently by their culturability the single-cell FCM analysis allows to discriminate bacteria from non-biotic particles with high precision disaggregation of biological floc was improved by optimising ultrasonication

– a procedure was proposed for converting the Forward Angle Light Scatter signal (FALS) produced by the viable or active bacteria into a correspondent cellular biovolume, adopting a calibration curve calculated with silica beads having size and refractive index similar to that of of bacteria cells (according to the Rayleigh-Gans theory). finally the number of bacteria cells is multiplied by the cellular biovolume and by the specif dry weigh, in order to obtain an equivalent cellular biomass.

The use of this integrated procedure based on FCM allowed to obtain fast analyses, completed in few minutes. The proposed method was applied to several samples of raw and pre-settled wastewater, activated sludge and effluents, with the aim to quantify viable and active biomass with respect to TSS content and to perform mass balances in wastewater treatment plants.

WILD GRAINS CHROMOSOMES IN SUSPENSION: FLOW KARYOTYPING AND SORTING OF DASYPYRUM VILLOSUM

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Flow cytogenetics, chromosome isolation, fluorescent in situ hybridization, wheat improvement

Dasypyrum villosum (*Dv*) is a typical wild species in the central-southern Italian peninsular and insular areas, where it grows in diversified environments (sublitoraneous calcareous sands, non-litoraneous sands or tuffs, scarce soil interspersed to calcareous rocks in semi-arid environments, cold mountainous terrain, disturbed road-sides) showing a polymorphic genome and an interesting ecological adaptations. *Dv* can be crossed with wheat and the introgression of its genes may contribute significantly to wheat improvement (i. e. genes for grain storage proteins and resistance to biotic and drought stresses).

In situ hybridization and molecular markers are useful methods to assess the introgression of *Dv* genes into wheat but crossing brings "good and bad" genes all together into the wheat genome, making a difficult task to obtain useful new varieties. Gene isolation and cloning for direct specific cisgenesis could be of most use to foster wheat improvement using specific genes from a related plant, but *Dv* genes have not jet been isolated.

Cytogenetics and flow sorting are relevant to speed up genetic characterization in *Dv* and to allow physical mapping, cloning and then direct transfer of useful genes thus avoiding traditional breeding drawbacks.

For the first time we have developed a complete system for cell cycle synchronization and chromosome isolation in suspension from fixed root tips in *Dv*. Root tip meristem cells underwent to double cell cycle synchronization with 1.25 mM hydroxyurea and 2.5 μM amiphrophosmethyl (APM) where meristematic cells reached a metaphase index higher than 50%. Root tips were fixed with 2% formaldeyde for 30 minutes and chromosomes suspension were produced after mechanical disruption at 13500rpm for 10sec in LB01 (Dolezel *et al.*,1989). About 10^5 ml^{-1} shaped chromosomes were isolated from 30 fixed root tips. Theoretical flow karytyping and real data showed that only a large chromosome could be isolated from a standard *Dv* complement.

COMBINING CARD-FISH and FLOW CYTOMETRY: NEW INSIGHTS and IMPROVEMENTS IN THE HYBRIDIZATION PROCEDURE

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Flow cytometry (FCM) and Fluorescence In Situ Hybridization (FISH) are widely used methods for the enumeration and identification of bacterial groups in environmental samples. In microbial ecology studies, many attempts have been made to combine the rapidity and accuracy of FCM with the specificity of fluorescent oligonucleotide probes. Several methodological problems (i.e. low fluorescent intensity, low hybridization efficiency, cell loss) still affect the reproducibility and variability of data. So far, the detachment of hybridized cells from filter membranes in a liquid suspension prior to flow cytometric analyses represents a promising approach, reporting high cell recovery efficiency (about 70%).

In this study, CARD-FISH was performed in a semi-closed system to further reduce hybridized cell loss. However, the cell clogged on filter membranes, probably due to the high density of hybridization and amplification buffers, represented the main methodological barrier. Cell detachment was therefore optimized comparing different detaching solutions and sonication times. Preliminary results showed high efficiency of cell detachment from filters after sonication in PBS (>90% of total SYBR Green I stained cells). Interestingly, the flow cytometric enumeration of EUB338 hybridized cell reached values ranging around 90% of total cells, which were comparable with values obtained by epifluorescence microscopic analysis.

TRACING VARIABILITY IN BEAUTY: PLOIDY EVALUATION OF A COLLECTION OF COMMERCIAL ORCHID HYBRIDS

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Flow cytometry; dna content; micropropagation; cattleya; phalenopysis; dendrobium.

Interspecific hybridization and chromosome doubling are techniques often applied to produce new cultivars of orchids. A better understanding of karyotypes and DNA contents of *Phalaenopsis, Dendrobium, Cattleya* will aid in the development of new cultivars that will improve the level and quality of production.

Endoreduplication is widespread in large numbers of higher plants. Sturdier flowers and better forms may accompany an increase in ploidy in orchids. Little is known regarding the mechanism of endoreduplication in orchids. Roots and stems of the hybrids were highly polysomatic. The patterns of polysomaty development were organ and developmental stage specific. Young leaves taken from in vitro plants showed a more stable DNA ploidy level and were a suitable material for determining the ploidy of orchid plants by flow cytometry. The nuclear DNA content of young invitro leaves of *Phalaenopsis amabilis*, *Cattleya intermedia*, *Cattleya Maxima*, *Phalaenopsis* Ching Ruey's "Black pearl" x "V. Light, *Phalaenopsis* "Violet light" x self, *Dendrobium phalaenopsis* (white)x *Dendrobium virgineum, Dendrobium phalaenopsis* (violet) x *Dendrobium loddigesi*, was measured by flow cytometry.

The early data obtained from these plants will be used to analyze genetic stability and to isolate somaclonal variants useful for orchid amelioration.

Use of FACS flow cytometry opens new possibilities for the genetic improvement of these genotypes through the sorting of enzimatically isolated polyploid protoplasts from spontaneous or colchicine induced endoreduplicated tissues.

COMPARISON OF METHODS TO DETECT DNA DAMAGE IN SPERM FROM DIFFERENT MAMMALIAN SPECIES

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Sperm DNA damage may have adverse effects on reproductive outcome. Sperm DNA breaks can be detected by a variety of in situ tests, the TUNEL assay, the Comet assay and the SCSA. These methods evaluate DNA integrity from different and complementary perspectives and offer a new class of biomarkers of the male reproductive function and of its possible impairment after environmental exposure, but their applicability needs to be evaluated.

The remodeling of sperm chromatin produces an extremely condensed nuclear structure which protects the nuclear genome from adverse environments. This remodeling is species-specific and differences in chromatin structure may lead to a dissimilar DNA susceptibility to a given stressor among species. In this study the capacity of SCSA, TUNEL and comet assay to detect DNA/chromatin integrity has been evaluated in human, mouse and bull sperm. The hypothesis that chromatin packaging might influence the amount of induced DNA damage was tested by treating sperm in vitro with DNAsel, the activity of which is strictly dependent upon its DNA accessibility. Furthermore, H_2O_2 was used to assess whether spermatozoa of the three species showed a different sensitivity to oxidative stress.

Results showed a different sensitivity to DNAsel treatment among the species with human sperm resulting the most susceptible. Reasonably, the more loosen structure of human sperm chromatin, characterized by a higher fraction of residual nucleosomal-like structure, makes DNA more accessible to the attack of the bulky DNAsel. On the contrary, no major differences among species were observed after H_2O_2 treatment. Furthermore, data obtained show a good correlation among the three tests in revealing sperm with DNA strand breaks. These results can be useful to standardize the protocols used to detect DNA damage in sperm, and provide helpful information for the characterization of reproductive hazard by a multitest experimental strategy and for the subsequent step of risk extrapolation to humans.

MODULATION OF CYTOKINES AND THEIR RECEPTORS IN HUMAN GLIOBLASTOMA CELLS AFTER IONIZING RADIATION

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The exposure of cells to ionising radiation has been reported to cause the release of several factors which are likely to be involved in some biological effects, such as genetic alterations, change in gene expression and lethality, occurring in the irradiated cells but also in the neighbouring non-irradiated cells (i.e. bystander effect). Cytokines are likely to be the molecules involved in the signal trasmission between irradiated and non-irradiated cells. In this study, we focussed our attention on IL-6 which contributes to malignant progression and apoptosis resistance of glioblastoma cells, on IL-8 that plays a role in promoting glioma growth and angiogenesis and on TGF-β which modulates autocrine glioma growth and invasion. We investigated the release modulation of these cytokines in the culture medium of human glioblastoma cells exposed to different doses (0-1Gy) of gamma radiation and the expression of the corresponding cell membrane receptors in irradiated cells and in cells cultured with medium collected and filtered from irradiated cells (bystander cells). We observed that cytokines are differently modulated by radiation. In particular, the release of IL-6 and IL-8 was significantly increased in a dose-dependent manner by gamma radiation at long time intervals (20 hours), whereas the release of IL-8 was decreased in irradiated cells (0.5 Gy) 5-7 hours after irradiation. TGF-β concentration was significantly increased in cells irradiated with 0.25Gy and 1Gy, 20 hours after exposure. In parallel, the immunocytochemistry analysis on the expression of their receptors in irradiated and bystander cells confirmed that the receptor profiles are modulated by radiation. In conclusion, our data suggest that these cytokines are likely

to play a role in the transmission of radiation-induced response, probably orchestrating the inflammatory microenvironment of the tumour. This highlights the importance of further studies aiming to investigate the pathways of intercellular communication involved in bystander phenomena also in view of applications for radiotherapy.

NEW GENETIC RESOURCES FOR WHEAT IMPROVEMENT BY FLOW BIOTECH: BETTER PROCEDURE TO CREATE ARM SPECIFIC BAC LIBRARIES FROM FEWER CHROMOSOMES

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Flow sorting; Triticum aestivum; cell cycle synchronization; genomics.

Genomics of large genomes is a challenging task, remarkably in plants where a large part of the nuclear DNA content (80 % or more) is made of repetitive DNA. Flow sorting of chomosomes in suspension is useful to dissect complex genomes to single chromosomes and chromosome arms, which represent a discrete and highly informative part of the whole genome, but accounting for only a few percent of the huge nuclear DNA content. The wheat genome (17Gbp for the haploid genome), one of the basic food source for the whole word, is a successful example for the application of flow biotechnology through the "chromosome approach". To date, 12 BAC libraries have been created from eight wheat chromosomes. They include a composite BAC library from chromosomes 1D, 4D and 6D, a BAC library from chromosome 3B, libraries from short and long arms of chromosomes 3A, 3D and 7D and a library from the short arm of chromosome 1B. The first generation of these resources had an average insert size below 100 kb as a consequence of a limited amount of DNA obtained after a time-consuming sorting, which enabled only one DNA size-selection step after partial digestion of chromosomal DNA. Recently we increased the efficiency of BAC cloning, which enabled the second size-selection step. The average insert size in the second generation chromosome BAC libraries exceeded 100 kb. These libraries provide over 10-fold chromosome coverage with only 30 – 50 thousand clones. A long-term goal of this work is to develop BAC libraries from each chromosome arm of the hexaploid wheat. Moreover, the increased cloning efficiency enabled production of custom BAC libraries constructed from a smaller number of chromosomes (~ 1 million) after a less stringent DNA size selection. Such libraries are useful in positional cloning of genes that are not present in cv. Chinese Spring, which has been used to develop a physical framework map of hexaploid wheat.

IN VITRO EFFECTS OF PHOTOACTIVATED MEROCYANINE-540 AGAINST S. EPIDERMIDIS BIOFILMS

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Bacterial infections are serious complications after medical device surgey. Staphylococci, with *Staphylococcus epidermidis* as a leading species, are the prevalent and most important species involved in orthopaedic implant-related infection. The biofilm mode of growth of these bacteria on an implant surface protects the organism from the host's immune system and from antibiotic therapy. Photodynamic therapy (PDT) is a promising new treatment modality for the inactivation of bacteria in biofilms. In PDT, light, O_2, and a photosensitizing drug are combined to produce a selective therapeutic effect.

In this study, we evaluated the antimicrobial activity of merocyanine 540 (MC 540), a photosensitizing dye that is used for purging malignant cells from autologous bone marrow grafts, against *Staphylococcus epidermidis* biofilms. Effect of the combined photodynamic action of MC 540 and 532 nm laser was investigated on the viability and structure of biofilms of two *Staphylococcus epidermidis* strains, RP62A and 1457.

Significant inactivation of cells was observed when biofilms were exposed to MC 540 and laser simultaneously. The effect was found to be light dose-dependent. Confocal laser scanning microscope (CLSM) analysis indicated damage to bacterial cell membranes in photodynamically treated biofilms. Furthermore the disappearance of MC 540 fluorescence observed in CLSM images of irradiated biofilms may be correlated to photobleaching of the dye.

ANTIBIOTIC ACTIVITY OF EXTRACTS FROM MEDITERRANEAN PLANTS EVALUATED BY FLOW CYTOMETRY ON BACILLUS SUBTILIS

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The antibiotic activity of natural compounds is often evaluated by using traditional techniques, based on bacteria proliferation on liquid or agarized media, which are time and labour- consuming. Flow cytometry offers real time microbial analysis without dependency of microbial culture and a rapid evaluation of bacterial detection and viability. In this study, flow cytometry was applied in order to evaluate the antibiotic activity of 10 Mediterranean plant extracts, obtained by using organic solvents, on *Bacillus subtilis*, a $Gram^+$ bacterium. Living cells were suspended in sterile buffer containing the different extracts (1%), and incubated overnight at 30 °C. Antibiotic and

methanol were used as positive and negative control, respectively.

After incubation, 5μl of dye solutions: thiazole orange (TO) and propidium iodide (PI) were added to 200μl of bacterial suspensions and further incubated for 15 minutes at room temperature. Data were analyzed using CellQuest software and for each sample, dead, viable and injured bacteria were distinguished by PI and TO respectively.

Extracts from *Lavandula sp*. and *Genista aspaathoides* showed high antibiotic activity on *B. subtilis*, whereas extracts from *Cistus sp*., *Erica scoparia* and *Inula viscose* showed no activity on bacterium.

Preliminary data on different activity of Mediterranean plant extracts on *B. subtilis* will be shown and the advantages of using flow cytometer techniques in comparison to traditional cultural tests will be discussed.

HEMATOLOGY

FLOW CYTOMETRY ANALYSIS OF CERBROSPINAL FLUID IN AGGRESSIVE LYMPHOMAS

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Cerebrospinal involvement is a frequent complication of hematological malignancies, with an incidence of up to 25% in certain leukaemias and lymphomas. The gold standard to detect cerebrospinal fluid (CSF) involvement is light microscopy. Unfortunately, this technique is characterized by low sensitivity and specificity. Secondary involvement of central nervous system (CNS) in aggressive non-Hodgkin lymphomas (NHL) is infrequent but often fatal. Clinical paradigms have been developed to identify patients at risk who may benefit from CNS prophylaxis. Since the cohort of patients at risk seems to be larger than the subgroup which will actually develop CNS disease, sensitive and specific laboratory methods are needed to detect occult CNS infiltration while ensuring optimal treatment and reducing unnecessary therapies.

The aim of this study was to assess the value of flow cytometry (FCM) in detecting CSF disease in patients with aggressive NHL, by comparing FCM results with cytologic findings.

Of 194 consecutive patients with newly diagnosed aggressive NHL, 69 (35%) were considered to be clinically at risk for CNS disease and underwent evaluation of CSF by FCM as well as light microscopy. FCM was able to detect an abnormal population, consistent with NHL infiltration, in 20 out of 69 patients (29%). Cytology detected abnormal cells only in 10 out of 69 patients (14%). These 10 patients were comprised in the cohort of 20 patients positive for FCM. These data indicate higher sensitivity of FCM in detecting CSF residing abnormal cells.

The results deriving from this study suggest that patients at risk for CNS involvement by aggressive NHL should always undergo staging evaluation of CSF by FCM. FCM positive patients, even in the presence of very low percentages of NHL cells, should be considered "true-risk" patient, candidate to receive active treatment.

ABERRANT GM-CSF SIGNAL TRANSDUCTION PATHWAY IN JUVENILE MYELOMONOCYTIC LEUKEMIA BY FLOW CYTOMETRY: RELIABILITY OF A RETROSPECTIVE STUDY

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Juvenile myelomonocytic leukemia (JMML) is a rare clonal myeloproliferative disorder of infancy and early childhood characterized by overproduction of myeloid cells (Aricò et al, *Blood*, 1997) and a selective hypersensitivity of the hematopoietic precursor cells to GM-CSF (Emanuel PD et al, *Blood*, 1991). Multiparametric flow cytometry analysis has demonstrated new potentialities for assaying intracellular levels of phosphorylated proteins (Irish JM et al, *Cell*, 2004). We and others have reported that *in vitro* response of JMML cells to GM-CSF demonstrated a greater increase in the % of STAT5-phosphorylated (p-STAT5) cells, in a single cell profiling assay, as compared to normal samples. (Gaipa G et al., *Leukemia*, 2008; Kotecha N et al., *Cancer Cell*, 2008).

In order to assess the feasibility of STAT5-phosphorylation also in thawed JMML samples, here we analyzed bone marrow (BM) mononuclear samples (5 fresh, 7 thawed) from 9 JMML patients at diagnosis and from 35 control subjects (17 fresh, 18 thawed). To characterize the immunophenotype of GM-CSF responding cells we applied either 3-colors (CD34/CD33/STAT5) or 7-colors (Live-Dead dye/CD33/CD34/CD14/CD45/STAT5/CD38) flow cytometry.

The aberrant p-STAT5 response was confirmed in CD34+/CD33+ cells from all fresh samples tested being clearly distinguishable from that of normsl controls, by contrast this was not found in thawed JMML samples bearing a lower p-STAT5 response. In particular, we noticed that STAT 5 expression was down modulated acccording to time delay from collection or time delay to freezing. However, when we evaluated the p-STAT5 expression by scaling the maximum response to 100%, as proposed by Kotecha et al. a clear resolution between JMML and controls could be

observed also in thawed samples. The extension of this approach to a larger series of JMML samples will help to investigate further biological insights of JMML and to evaluate to what extent this new assay may be considered as a tool for the diagnostic work-up of JMML patients.

CD22, CD79b, CD81 AND CD200 HIGHLY SPECIFIC MARKERS CAN ENHANCE THE FLOW CYTOMERIC (FC) DIAGNOSIS AND MONITORING OF B-CLL AND B-NHL

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FC diagnosis of B-NHL is challenging. Even CLL, the most common low grade disorder, can be misdiagnosed, especially when CD5 or CD23 are present at low density or weak FMC7 staining is observed. For this reason, new markers (CD200) or disease-specific antibody combinations (CD81/CD22/CD79b on CD19/CD5 B cells) have been recently identified for CLL immunophenotypic characterisation and MRD monitoring.

We have introduced the newly described antibodies in our FC practice to improve immunophenotypic profile definition, differential diagnosis and MRD detection in CLL and other B-NHL.

PB, BMA and pleural effusion samples from 63 consecutive B-NHL patients (46 CLL, 4 FL, 4 MCL, 4 MZL, 1 HCL, 1 LPC, 3 BL) were tested with CD79b, CD200 and the CD81/CD22 antibody combination.

CD22 and CD81 were downmodulated in all but 4 CLL and in 2/4 MCL. Likewise, CD81 was weak in 1/1 HCL, 2/4 MZL and 1/4 FL. Interestingly, in 2/3 BL and in 1 transformed FL, CD81 was up-regulated to levels comparable to immature B-cells. In 20 patients (CLL, MZL, LPC), the CD81/CD22 combination was used for highly sensitive and specific monitoring of MRD. As expected, CD79b was undetectable or dim in CLL, while CD200 was highly expressed in all but 2 CLL and in 1/1 HCL; surprisingly, bright CD200 was also found in 2 MCL. Conversely, 2/4 FL, 4/4 MZL, 1/3 MCL and 3/3 BL turned out CD200-. According to the FC data, histological re-evaluation and molecular investigation were performed in 3 cases (1 CLL and 2 MCL), allowing correct re-classification.

These data confirm that the CD81, CD22, CD79b represent a powerful tool for the immunophenotypic characterisation and follow-up monitoring of CLL. Likewise, if confirmed on a larger series of cases, CD200 may represent an interesting marker for the differential diagnosis of both low and high grade B-NHL, with possible clinical implication in the field of target therapies.

MULTIPARAMETRIC FLOW CYTOMETRIC CHARACTERIZATION OF CIRCULATING STEM CELL SUBSETS IN PATIENTS WITH MIOCARDIAL INFARCTION: CORRELATION WITH CLINICAL AND BIOLOGICAL DATA

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In an attempt to better understand the functional and immunophenotypic characteristics of circulating stem cell subsets we investigated and analysed 120 patients from 1st to the 6th days after acute MI. The presence of the different stem cell subsets were correlated to the in vitro functional characteristics of hematopoietic/endothelial progenitor cells and to the different patient's clinical parameters to clarify which could be regarded as the most predictive of long-term clinical follow-up. The multiparametric flow cytometric protocol was necessary to overcome immunophenotypic misunderstanding since the different hemopoietic and endothelial stem cells populations share numerous surfaces markers.

The analysis of the CD34+ cells (ranging from 0,024 to 0,037%) subsets showed that CD34+cells defined by the expression of in particular the CD117 (c-kit) or CD146 markers are higher after five days of MI. No significative number of circulating EPC were observed. Short term clonogenic assay showed an high number of in vitro generated BFU-E (burst forming unit erithroblasts) at five days after MI that correlated with the percentage of the circulating CD117+ and CD146+ CD34+CD45+ stem cells. The presence of in vitro EPC colonies was found only ten days after MI in 3% of patients only. On the other hand, 52% of patients showed the presence of in vitro endothelial-like monocytic colonies 5 days after MI. Patients displaying high number of BFU-E resulted characterized by no cardiovascular events at nearly 6 months follow up, and this may represent an important role in myocardial repair. All these patients had no smoking habits, and this data confirm previous studies results in which stem cells mobilization was lower in smoking subjects. In the same group of patients there was an higher number of diseased vessels. These data would be useful for clinical setting.

This study was supported by Programma di Ricerca medicina Rigenerativa Regione Emilia Romagna-Università 2007-2009.

IMMUNOPHENOTYPIC HETEROGENEITY OF MESENCHYMAL STROMAL CELLS: MULTIPARAMETRIC FLOW CYTOMETRIC ANALYSIS

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So far, the immunophenotypic profile of freshly isolated and ex-vivo expanded human mesenchymal stromal

cells (hMSCs) has been confined to single or dual staining analysis especially in normal subjects. The lack of specific markers complicate the in vivo hMSC detection. The immunophenotype of cultured hMSC is not still elucidated. Although hMSCs are reported to be uniformely positive for CD90, CD105, CD73, we specifically investigated the hMSC immunophenotype after ex vivo expansion in relation to different parameters such as different culture conditions (serum free, additional use of platelet-lysate, cytokines), culture age, hMSC source (different tissues and normal versus pathologic donors, such as hematological malignancies, HM) and contaminant CD45pos hematopoietic cells and/or endothelial cells. The human primary MSC immunophenotype was also compared to that of different transformed tumor cell lines phenotype. Based on these observations, we observed hMSC immunophenotypic modulation in particular of CD44, CD10, CD146 in relation to the hMSC source and culture passages.

The downregulation of CD90 expression is documented on bone marrow-derived hMSC treated with angiogenic cytokines and in some HM patients and is also related to a diminished immunomodulant MSC capacity. Of interest is the expression of the CD34 stem cell marker that is universally known to be negative on hMSC but that we found to be positive uniquely on hMSC from lipoaspirates.

The lack of "MSC" markers such as CD90, CD73, CD106 and CD146 expression was observed on some cancer cell lines from colon and epatocarcinomas. The results confirm an immunophenotypic heterogeneity of cultured hMSC that could have different clinical implications. The study of hMSC cell immunophenotypic subsets could be useful before their use in transplantation setting.

ELEVATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND STROMAL DERIVED FACTOR AFTER MYOCARDIAL INJURY MOBILIZES CD34+ CELLS IN CARDIOVASCULAR PATIENTS

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Objective: it is not known whether bone marrow cells (BMC) mobilization in human is triggered by necrosis, ischemia or both. Recognition of the trigger is important to improve therapeutic use of BMC. The aim of this work is to test the role of necrosis, ischemia or both in BMC mobilization in patients with cardiovascular disease. Methods: we studied three groups of patients (P) : group 1 with pure ischemia (24 P with unstable angina); group 2 with pure necrosis (28 P undergoing transcatheter radiofrequency ablation, without CAD); group 3 with ischemia + necrosis (30 P with transmural myocardial infarction). As control groups we studied 27 P with angiographically documented stable angina (C1), and 20 P without CAD undergoing coronary artery angiography for valvular diseases or cardiomiopathy (C2). CD34+ cells and cytokines were monitorated at: T_0 (baseline), 48 hours and 5, 7, 10, 14 days thereafter. **Results**. In the groups with necrosis (1 and 2), there was a significant increase of CD34+ cells at T_3 and T_4 (after 7 and 10 days, respectively). The peak of mobilization was observed ten days after the necrotic event (2.8 ± 1.4 vs. 5.9 ± 1.9 in the group 1, $p=0.03$; and 3 ± 1.5 vs. 5.6 ± 2 in the group 2, $p=0.04$; respectively). There was a correlation between CD34+ and SDF and VEGF peak values ($r=0.77$ and $r=0.63$, respectively), but no correlation between peaks of CK-MB or troponin and CD34+. **Conclusions**: necrosis, but not ischemia, causes an increase of VEGF and SDF1and a CD34 mobilization. CD34 mobilitation is not correlated to the extension of necrosis.

This study was supported by Programma di Ricerca medicina Rigenerativa Regione Emilia Romagna-Università 2007-2009.

MULTIPARAMETER IMMUNOPHENOTYPING BY FLOW CYTOMETRY IN MULTIPLE MYELOMA: DEFINING RANGES OF NORMAL EXPRESSION AND THEIR DIAGNOSTIC UTILITY

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Background: In order to appropriately study multiple myeloma (MM) utilizing flow cytometry (FC) it is necessary to be able to distinguish between the normal and abnormal plasma cells (PCs). Numerous studies have reported on the immunophenotype of PC cell neoplasms, but very few have examined the immunophenotype of normal PCs. In this study, an objective definition of normal range of expression for each antigen was found on normal control PC obtained from orthopaedic resections. Using these new ranges of normal expression (new method) is different from using a static 20% cut-off described in the literature (traditional method0. These newly calculated normal ranges for each antigen were applied to patients' data, and compared to histologic and immunohistochemical findings.

Methods: Bone marrow samples from 55 patients with plasma cell neoplasms and 15 normal controls were studied. A minimum of 100 PC were analyzed for each patient and control sample. An 8-color staining method was applied to study the immunophenotype of PCs, using a BD FACSCanto II.

Results: CD19 correlated with histology by both the traditional and new methods, but had superior correlation by the new method.

Conclusions: This report is the first 8-color immunophenotypic study of MM in which a "range of normal expression" for each antigen is defined. This is a critical step to discern which PCs antigens are of diagnostic importance.

PERIPHERAL T CELL LYMPHOMA ASSOCIATED WITH MYELOFIBROSIS: A CASE REPORT

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Background: Bone marrow fibrosis is associated with myeloproliferative disorders and hairy cell leukemia. Furthermore, myelofibrosis occurs in metastatic carcinoma, mycobacterial infections, systemic lupus erythematosus and various other disorders. Approximately 8-30% of patients with multiple myeloma present a fibrotic bone marrow. However there are only few reports of myelofibrosis associated with B and T lymphomas. We report a case of an unspecified peripheral T-cell lymphoma coexpressing CD4 and CD8 and presenting as pancytopenia due to fibrosis of bone marrow.

Case report: A 67–year-old female was admitted to our hospital for fever, cough and weight loss. There was no evidence of lymphadenopathy or hepatosplenomegaly. A complete blood count showed pancytopenia but also an increased absolute lymphocytic count. A bone marrow biopsy revealed diffuse fibrotic change with an increased percentage of megakaryoblasts. Immunophenotyping revealed an increased percentage of a T cell population coexpressing CD4 and CD8. Polymerase chain reaction (PCR) analysis showed the gene rearrangement of T-cell receptor. A diagnosis of an indolent peripheral T-cell lymphoma with myelofibrosis was made. The patient underwent therapy which improved her pancytopenia while lymphocytic count came back within normal ranges.

Conclusion: This case report shows that myelofibrosis and T-cell lymphoma can coexist in the same patient. The increased percentage of megacaryocytes could be implicated as the source of the cytokines that may augment fibroblast proliferation. However, whether myelofibrosis and T-cell lymphoma have a common pathogenesis or whether one disease is consequent to the other one is unclear.

THE DIAGNOSTIC ROLE OF MULTIPARAMETER IMMUNOPHENOTYPING BY FLOW CYTOMETRY IN MULTIPLE MYELOMA: A NEW MODEL

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Background: Multiparameter flow cytometry (FC) represents an attractive approach in the detection of abnormal plasma cells (aPC) in Multiple Myeloma (MM) due to its capacity to combine an examination of both immunophenotype and clonality. Due to the large numbers of cells amenable to analysis by FC, it may be additionally useful in the detection of minimal residual disease (MRD). Problems with such evaluation of PC include those related to the frequent hemo-dilution of bone marrow aspirates (BMA) with peripheral blood (PB) as well as the liability of PC stored outside of the body. The histologic examination of BM remains the gold standard in the diagnosis of MM. We have developed a new statistical diagnostic model that examines what correlation exists between the immunophenotype and clonality detected by FC and histology, defining the diagnostic role of FC in MM.

Methods: 55 patients were enrolled in a pilot study for routine diagnostic analysis of MM; a minimum of 100 PC were analyzed for each patient sample. A direct 8-color method was applied to study the immunophenotype of PC, utilizing a BD FACSCanto II.

Results: CD38, CD19 and CD10 expression, when applied to our model, resulted in optimal concordance with histology.

Conclusions: This statistical model showed a correlation between FC and histology. It represents a new objective and reproducible way to interpret the immunophenotype of PC and correlates this analysis with histological results. Our goal is to use this information to consolidate this model and test its applicability on a larger scale.

ABNORMAL PHENOTYPE OF BONE MARROW PLASMA CELLS FROM PATIENTS TREATED WITH IMATINIB FOR CHRONIC MYELOID LEUKEMIA

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Imatinib is the initial therapy of chronic myeloid leukemia (CML) and induces several effects on immune system, including hypogammaglobulinemia. Our aim was to evaluate a possible interference of imatinib with plasma cell phenotype.

Thirty CML patients, undergoing imatinib therapy were evaluated. Bone marrow samples were collected at the time of the monitoring of response to therapy. Flow cytometry was performed by a 6-fluorescence method, using a FacsCanto II cytometer and a MoAb combination with CD138, CD38, CD19, CD45, CD117, CD56 and CD27. 500,000 events/tube were acquired. Plasma cells were identified as CD138+CD38+ and were defined as normal when CD19 and CD45 were both positive. Ten subjects, including normal individuals and patients with other chronic myeloproliferative diseases, were evaluated as controls. Plasma protein electrophoresis, g-globulin levels and serum immunofixation were always registered.

9 patients showed a normal plasma cell phenotype, similar to controls (CD19+CD45+CD27+CD56-CD117-). Bone marrow samples from the remaining 21 patients showed $>$ 20% (49 ± 17, range 25-100) abnormal plasma cells always CD19-CD45-. The abnormal plasma cell popula-

tions were CD117- and CD27+, but in 12 cases CD56 was found to be co-expressed by 25-52% of cells. A monoclonal component was never found.

Therapy with imatinib induces a plasma cell subpopulation phenotypically abnormal without, however, any correlation with g-globulin levels.

FLOW CYTOMETRIC ANALYSIS OF CD135 MEMBRANE EXPRESSION IN ACUTE MYELOID LEUKEMIA

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Background: The class III receptor tyrosine kinase FLT3/FLK2 (FLT3; CD135) represents a crucial molecule involved in early steps of hematopoiesis. In addition, it has widely been proved that FLT3 mutations in Acute Myeloid Leukemia (AML) are significantly associated with unfavourable prognosis. The aim of this study is to verify the role of FLT3 tyrosine kinase receptor (CD135) expressed by leukemic cells of patients with AML and to correlate it with FLT3 molecular expression and other biological and clinical parameters. Patients and Methods: The membrane expression of CD135 has been analysed by flow cytometry in 42 patients with AML (M/F 28/14: median age 64 - range 27-84-; FAB M1/M2: 20; FAB M4/M5 22). The results have been correlated with the molecular expression of FLT3 mutation, with the bone marrow and the peripheral blood leukemic involvement, with FAB cytotype and with the immunophenotype including the surface expression of CD34, CD117 CD56 antigens. Results: Out of the 42 patients tested, only 14 cases showed that the membrane expression of CD135 resulted less than 20%. Both the bone marrow infiltration (67% vs 44%: p 0.01) and the peripheral leukocytosis (41 vs 12 $\times$10(e)8/L: $p < 0.01$) were largely found in cases that showed CD135 expression. The results obtained with the flow cytometric analysis almost completely overlapped the results obtained by molecular analysis: genetic mutation was absent in the CD135 negative cases, whereas it was present in 86% (24/28) of CD135 positive cases. Overall, CD135 positive expression was found in 82% (18/22) of FAB M4/M5 AML and only in 50% (10/20) of FAB M1/M2 AML. Both the expression of CD34, CD117, CD56 and T and B lymphoid cell lines antigens resulted completely independent to the CD135 expression. Conclusions: According to our experience, the membrane antigenic expression of FLT3 receptor has represented: a) a high correlation marker with the AML aggressiveness evaluated both with bone marrow and peripheral blood blastosis; b) a marker significantly related to the cytotypes M4 and M5 AML of FAB classification; c) an indipendent marker from the expression of stem cell antigens or of B and T cell lines antigens; d) a marker closely related to the FLT3 genetic mutation. This last above mentioned characteristic describes the membrane antigenic expression of FLT3 receptor as a biologically and clinically valid parameter, easily replaceable to the molecular analysis in the AML prognostic evaluation.

DUAL INHIBITION OF PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) AND mTOR AS A NEW THERAPEUTIC OPTION FOR T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL)

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Constitutively activated PI3K/Akt/mTOR signaling is a common feature of T-ALL, where it contributes to cell proliferation and survival. These findings lend compelling weight for the application of PI3K/Akt/mTOR inhibitors in T-ALL. Here, we have analyzed the therapeutic potential of the dual PI3K/mTOR inhibitor, BEZ235 (Axon Medchem BV), an orally bioavailable imidazoquinoline derivative, which has entered clinical trials for solid tumors. BEZ235 was cytotoxic to a panel of T-ALL cell lines including 170-kDa P-gp overexpressing cells, in a IC_{50} range from 70 to 200 nM at 24 h. BEZ235 induced a G1 phase cell cycle arrest and apoptotic cell death accompanied by dephosphorylation of Akt, GSK3β, p70S6K, ribosomal S6 protein and 4E-BP1. Remarkably, BEZ235 targeted the SP (identified by Hoechst 33342 staining) of T-ALL cell lines, which might correspond to leukemia initiating cells, and synergized with chemotherapeutic drugs (dexamethasone, vincristine, cyclophosphamide). Our data indicate that multitargeted therapy towards PI3K and mTOR, may serve as an efficient treatment towards T-ALL cells which require upregulation of PI3K/Akt/mTOR signaling for their growth/survival.

REGULATORY T-CELLS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Naturally arising CD25+CD4+ regulatory T-cells (Treg) actively maintain immunological self-tolerance. Deficiency in/or deficiency of these cells can be a cause of autoimmune disease. A reduction in their number of function can also elicit tumor immunity. Several studies evidenced that the immune system in patients with chronic lymphocytic leukemia (CLL) is deficient. In the current study we have evaluated, by means of a multiparametric flow cytometric approach, the peripheral blood Treg number in 15 patients with untreated CLL (8 male, 8 female, mean age 69 years, range 62-82 years) and in 15 normal subjects (8 male and 8 female, mean age 56 years; range 39-67 years).

CD4+CD25+high density cells were gated and evaluated for CD127 positivity at low density to analyze only Treg. As expected, the white blood cell count and absolute lymphocyte count was found higher in CLL patients (mean number 29.700/ mL, range 7.900-73.300/mL and 23.347/ mL, range 5000 - 66.900/ mL, respectively) with respect to healthy volunteers (mean number 6.358/ mL, range

4.300-9.600/ mL and 2.450/ mL, range 2.000-2.800/ mL). Treg were detected at lower percentage number in CLL patients (mean number 0.16%, range 0-0,3%) than in controls (1.09%, range 0.2-2.4%). On the contrary, when evaluated as absolute number, CLL patients showed a higher number of Treg (mean number 75.7/ mL, range 0-344/mL) compared to controls (mean number 26.4/ mL, range 5-56.7/mL). In both cohort of samples CD25+high density cells showed the expression of CD127 at low density. Only in a patient with CLL Treg were found undetectable. This patient suffered from an autoimmune complication of CLL (autoimmune hemolytic anemia) at the moment of analysis. In conclusion, our data, despite preliminary and limited in number, showed that Treg are higher in CLL patients. This subset of cells is probably involved in the crucial mechanism of pathogenesis of the disease. Moreover, a reduced number of Treg has been reported to allow the emergence of autoimmune disorders

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CYTOMETRIC IMMUNOBEAD ASSAY FOR THE DETECTION OF BCR-ABL PROTEIN: ITS POTENTIAL USE FOR MINIMAL RESIDUAL DISEASE EVALUATION

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Here we describe the potential application of the BD BCR-ABL Protein Kit to the evaluation of minimal residual disease (MRD). In brief, intact cells were pretreated with protease inhibitors, spun down and immediately lysed. Each sample was incubated for 2 hours with capture beads and detector reagent to allow the formation of the "sandwich" complex where the bead-bound antibody recognizes the BCR component and the phycoerythrin (PE)-conjugated antibody recognizes the ABL part. Samples were analyzed by BD FACSCanto II and FACS-Diva software. The analytical detection limit, i.e. the minimum PE MFI value characterizing positive samples, was 66.2+26 MFI (mean MFI plus 2 SD of 9 BCR-ABL negative bone marrow [BM] samples). In order to test the sensitivity of the assay, we diluted the BCR-ABL+ cell line K-562 into normal BM at progressively decreasing concentrations. We found that BCR-ABL associated fluorescence signals were distinctly detected at the concentration of 0.1% K-562. In order to investigate the ability of beads to bind even low fusion protein amounts while preserving bright fluorescence, we set up a 'miniaturized' assay by using only 5×10^4 K-562 cells (instead of 2.5×10^6) and a ten-fold decreased amount of beads. As expected, we found smaller clusters of beads within the dot-plots, but bright BCR-ABL positivity (>4.000 MFI).

Our data suggest that: (i) the BCR-ABL Protein Kit shows a baseline sensitivity of 10^{-3}; (ii) it is possible to miniaturize the test by using decreased numbers of target cells and beads, thus obtaining good fluorescence signal in spite of low amounts of protein. These data indicate that the assay is sufficiently sensitive to detect MRD even at percentages markedly lower than 10^{-3}. New experiments are in progress based upon the use of cell sorting isolation of CD19+ cells in Ph+ ALL followed by the 'miniaturized' BCR-ABL protein assay.

FLOW CYTOMETRY EVALUATION OF ZAP-70, CD38 AND CD49D ANTIGEN EXPRESSION ON THE NEOPLASTIC CELLS OF PROGRESSIVE CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Background: In recent years, different biological features have been associated with the prognosis and clinical course of chronic lymphocytic leukemia (CLL) patients. In particular, the expression of the ZAP-70, CD38, CD49d molecules on CLL cells bears prognostic implications being associated with an unfavorable outcome; the flow cytometric detection of such molecules is of relatively simple execution. Likewise, the unmutated immunoglobulin heavy chain variable region gene (UM-IGHV) status has been also associated with an aggressive disease.

Methods: In 45 patients with CLL at the time of starting 1st line treatment because of disease progression, ZAP-70, CD38 andCD49d were examined by flow cytometry and their expression was correlated with the IGHV mutation status analyzed by sequencing. The cytoplasmic expression of ZAP-70 ($\geq$20%) was assessed using the Alexa fluor 488-conjugated anti-ZAP-70 MoAb (Caltag Laboratories, Carlsbad, CA), while the expression of CD38 ($\geq$20%) and CD49d ($\geq$30%) were detected using phycoerithrin (PE)-conjugated anti-CD38 and CD49d MoAb, respectively (BD, Biosciences, San Jose, CA). The analysis were performed using the FACS-Canto flow cytometer (BD).

Results: Forty-one of the 45 cases analyzed (91%) expressed the ZAP-70 molecule, while 67% showed an UM-IGHV status; 20/45 cases (44%) expressed the CD38 antigen and 87% showed an UM-IGHV status; 21/45 cases (46%) expressed CD49d and 76% were unmutated. The expression of CD38 and CD49d was concordant in 71% of cases: both antigens were positive in 31% of cases and both were negative in 40%. Both ZAP-70 and CD38 were expressed in 49% of patients. Co-expression of the 3 antigens was present in 14 of the 45 cases, 83% of which showed an UM-IGHV status. No single case failed to express at least one of the above three antigens.

Conclusions: All progressive patients evaluated at the time of 1st line treatment showed the expression of at least one of the poor prognostic factors ZAP-70, CD38 and CD49d evaluated by flow cytometry. The ZAP-70 molecule was positive in 90% of the cases representing the most reliable prognostic factor associated with progressive disease. In no case was the absence of all three antigens recorded. Additional prospective studies are needed to further clarify the role of these antigens as predictors of response to therapy in patients with CLL.

RELATIONSHIP BETWEEN CD49d AND CD38 EXPRESSION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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CD49d (VLA-4) is an adhesion molecule that represents a novel prognostic marker for Chronic Lymphocytic Leukemia (CLL). CD38 is a marker of proliferating CLL cells. The expression of these two antigens is reported as a progressive disease indicator. Between July 2008 and May 2009 we investigated 160 specimens from patients with lymphoproliferative disorders by flow cytometry analysis. 63 patients had typical CLL expressing CD19 CD5 CD23 and weak, clonally restricted surface immunoglobulin. Immunophenotyping of these patients was performed by the flow cytometer FACSCanto II (Becton Dickinson) by using a 6-colour strategy. The panel of antibodies included reagents specific for CD3 CD5 CD19 CD20 CD22 CD23 CD103 CD25 CD37 CD11c CD10 FMC7 HLA-DR CD43 CD49d CD38 CD45. In our study, we evaluated the relationship between CD49d and CD38 expression on CD19+/CD5+/CD23+ cells. Results of CD49d and CD38 expression were reported as mean of fluorescence intensity (MFI). In 45 cases (71.4%) there was a direct correlation between CD49d and CD38 expression. Among these, 27 patients (42.9%) were CD49d-/CD38-, while 18 patients (28.5%) were CD49d+/CD38+. By contrast, 18 cases (28.6%) showed a discordant expression in that 16 patients (25.4%) were CD49d+/CD38- and 2 (3.2%) were found CD49d-/CD38+. Finally, CD49d was found strongly expressed on polyclonal residual B lymphocytes when compared to CD19+/CD5+/CD23+ CLL cells. This seems plausible given that CD49d has an important role as a modulator of intracellular signaling and inhibits apoptosis in normal mature B cells.

In conclusion, CD49d seems to be expressed on normal circulating B cells and to split CLL patients into two subpopulations. The significance of the simultaneous use of CD38 and CD49d in CLL prognostic assessment remains to be elucidated.

THE FLOW CYTOMETRIC PATTERN OF CD10 AND BCL-2 EXPRESSIONS IS A USEFUL TOOL TO IDENTIFY FOLLICULAR LYMPHOMA CELLS

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Follicular lymphoma (FL) is a specific entity defined by characteristic histology, phenotype and molecular rearrangements. Classically reactivity for CD19 CD10 and strong positivity for the surface light chain immunoglobulin are considered to be phenotypic signs typically expressed in FL. CD10 is expressed on the vast majority of FL cells and in a subset of diffuse large B-cell lymphomas (DLBCL). The bcl-2 oncoprotein, a 26-kd protein that prolongs cell survival by inhibiting apoptosis, has been a particularly useful target for distinguishing FL from other lymphomas. We utilized 6-colour flow cytometry strategy using a FACSCanto II (Becton-Dickinson). The panel of antibodies included reagents specific for CD3 CD5 CD19 CD20 CD22 CD23 CD103 CD25 CD37 CD11c CD10 FMC7 HLA-DR CD43 CD49d CD38 CD45. In addition, a combined surface CD3/CD10/CD19/CD45 and intracellular bcl-2 staining was performed. We reported six cases suspected as FL. Bone marrow and peripheral blood from four patients showed high intensity of CD10 and strong bcl-2 expression, this expression was higher than that of any cell subpopulation, confirming the diagnosis of FL. In contrast, two patients demonstrated the same bcl-2 expression as CD3+ T cells and low intensity of CD10, related to DLBCL. Flow cytometry strategy has the advantage of being highly quantitative, thus our results show that the analysis of bcl-2 and CD10 expressions by flow cytometry adds an additional piece of confirmatory data that, in difficult or inconclusive cases, can help to establish the diagnosis of FL.

THE IMMUNOGLOBULIN GENE REPERTOIRE OF LOW-COUNT CLL-LIKE MBL IS DIFFERENT FROM CLL: DIAGNOSTIC IMPLICATIONS FOR CLINICAL MONITORING

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The term Monoclonal B Lymphocytosis (MBL) defines the presence of monoclonal B cells in the blood of otherwise healthy individuals. Though phenotypically heterogeneous, most MBL cases resemble CLL cells ($CD5^{+}$, $CD20^{dim}$, $CD79b^{dim}$, sIg^{dim}). The interest in MBL increased after this entity was included in the revised NCI-WG/IWCLL guidelines for the diagnosis and management of CLL and defined as "the presence of fewer than 5×10^{9}/L of B lymphocytes" in the peripheral blood. MBL in subjects with lymphocytosis requires treatment at a rate of 1.1% per year and presents immunoglobulin (IG) gene features and cytogenetic abnormalities similar to good prognosis CLL. That notwithstanding, the concentration of MBL in the blood may be extremely variable, and it is plausible that cases with high-count MBL are likely more advanced on the way to become CLL. In order to dissect molecular differences that could potentially distinguish between high-count MBL and low-count cases present in the general population, we studied, by cytofluorograph analysis, the blood of 1725 healthy individuals >18 years old. We identified 89 CLL-like MBL (5.1%), that represented in most cases a minority of all circulating B lymphocytes (low-count MBL) and we analyzed the expressed IGHV-D-J rearrangements in 51 cases. The 70 % of the IGHV genes were mutated and the most frequent IGHV genes was IGHV4-59/61, rarely used in CLL, while the IGHV1-69 and IGHV4-34 gene were either absent or infrequent. Only 2/51 (3.9%) MBL cases expressed a CLL-specific stereotyped HCDR3. These results show that the IG gene repertoire in low-count MBL differs from both mutated and unmutated CLL, suggest-

ing that the detection of MBL in an otherwise healthy subject is not always equivalent to a pre-leukemic state and a detailed molecular analysis may help to define the risk of potential disease progression.

MITOCHONDRIA REGULATE PLATELET METAMORPHOSIS INDUCED BY OPSONIZED ZYMOSAN A: ACTIVATION AND LONG TERM COMMITMENT TO CELL DEATH

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Changes of mitochondrial membrane potential play a key role in determining cell fate. Mitochondria membrane hyperpolarization has in fact been found after cell activation, e.g., in lymphocytes, whereas depolarization has been associated with apoptosis execution. The aim of this study was to investigate the effects of an immunologic stimulus, i.e. opsonized zymosan A, on human platelet mitochondria by means of flow and static cytometry analyses as well as by biochemical methods.

We found that opsonized zymosan induced, at early time points (90 minutes), significant changes of platelet morphology. This was associated with increased reactive oxygen species production and, intriguingly, mitochondrial membrane hyperpolarization. Later (24 hours) opsonized zymosan induced: i) increased CD47 adhesion molecule expression, ii) platelet aggregation; iii) mitochondrial membrane depolarization, and iv) phosphatidylserine externalization. Although in nucleated cells these late events usually represent signs of apoptosis execution, in opsonized zymosan-treated platelets they were not associated with membrane integrity loss, changes of Bcl-2 family protein expression and caspase activation. In addition, pre-treatment with low doses of the "mitochondriotropic" protonophore carbonyl cyanide p-(trifluoro-methoxy) phenylhydrazone counteracted mitochondrial membrane potential alterations, reactive oxygen species production and phosphatidylserine externalization induced by opsonized zymosan.

Our data suggest that: i) mitochondrial hyperpolarization can represent a key event in platelet activation and remodeling under opsonized zymosan immunological stimulation and ii) opsonized zymosan immunological stimulation may represent a useful tool for the understanding of the pathogenetic role of platelet alterations associated to vascular complications occurring in metabolic and autoimmune diseases.

A CASE OF PLATELET-TYPE VON WILLEBRAND DISEASE: DIAGNOSTIC VALUE OF FLOW CYTOMETRY

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Platelet-type von Willebrand disease (PT-VWD) is a rare autosomal dominant bleeding disorder due to a mutation in the gene encoding for platelet glycoprotein Ibα(GPIbα) leading to an enhanced affinity of GPIbα for von Willebrand factor (VWF). Platelets bind spontaneously high molecular weight (HMW) multimers of VWF and are cleared from the circulation resulting in thrombocytopenia and loss of HMW VWF multimers. Laboratory features resemble those of type 2B VWD, due to a mutation in the gene encoding for VWF. The differential diagnosis of the two diseases is important to choose the appropriate treatment. We have characterized a case of PT-VWD and evaluated the usefulness of a new flow cytometric diagnostic test we have recently described for the evaluation of VWF binding to platelets, in the differential diagnosis of PT-VWD and type 2B VWD. Patient had a prolonged bleeding time, a mildly reduced platelet count, and a reduced ratio VWF activity/antigen. Ristocetin induced platelet aggregation (RIPA) and VWF binding to platelets, evaluated by flow cytometry, were markedly increased. The addition of cryoprecipitate induced platelet aggregation and VWF binding to platelets of the patient with PT-VWD but not of a patient with type 2B VWD. Aggregometric and flow cytometric mixing tests highlighted the platelet origin of the defect while confirmed the plasmatic defect in a patient with type 2B VWD. Genetic analysis revealed a heterozygous point mutation in codon 239 previously described in association with PT-VWD. In conclusion the flow cytometric assay we described was able to highlight the increased affinity of VWF for GPIbα in the same way as RIPA and, when applied to mixing tests, to differentiate PT-VWD from type 2B VWD. Flow cytometry may become a useful tool for the diagnosis of VWD and for the discrimination of different VWD types including type 2B and PT-VWD.

FLOW CYTOMETRIC ANALYSIS OF FINE NEEDLE ASPIRATION CITOLOGY (FNAC) IN NON HODGKIN LYMPHOMAS

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Flow cytometry (FC) is a useful adjunct to fine-needle aspiration cytology (FNAC) in evaluating lymphoproliferative disorders. We reported a study of 383 FNAC from lymph nodes. We utilized 6-colour flow cytometry strategy using a FACSCanto II (Becton Dickinson). The panel of antibodies included reagents specific for CD3 CD5 CD19 CD20 CD22 CD23 CD103 CD25 CD37 CD11c CD10 FMC7 HLA-DR CD43 CD49d CD38 CD45 and bcl-2. The samples were classified within immunophenotypic pattern : 15 inadequate, 20 suspicious, 178 benign reactive hyperplasias (BRHs) were CD19+ CD20+ and both kappa and lambda light chains positive, 170 primary non-Hodgkin lymphomas (NHLs). Among these NHLs, 162 showed positivity against B-cell antigens CD19 CD20 and monoclonality for kappa or lambda light chains and 8 NHL patients expressed T-cell antigens CD7 CD1a. 119 were classified as diffuse large B-cell lymphomas (DLBCL), 8 included follicular lymphomas (FL) CD10+ bcl-2++, 30 were mantle cell lymphomas (MCL) CD5+ CD23- CD22+, 3 were defined as

chronic lymphocytic leukemias (CLL) CD5+ CD23+, 1 Burkitt lymphoma and 1 NK lymphoma. FC/FNAC diagnoses were confirmed histologically. FC applied to FNAC enhanced the precision of cytologic diagnosis in lymph nodal and extra lymph nodal lymphoproliferative disorders and allowed further subclassification in more than half of the cases, thus avoiding invasive surgical biopsies in many patients. The combination of FNAC and flow cytometry obtained by FNAC can distinguish between benign and malignant lymphoid infiltrates and supports a diagnosis of lymphoma.

MINIMAL RESIDUAL DISEASE PROGRESSION CHART: AN ADDITIONAL INSTRUMENT TO GET AN OVERALL VIEW OF THE FOLLOW UP IN ACUTE MYELOID LEUKEMIA

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It is expected that the quantification of minimal residual disease (MRD) significantly contributes to the assessment of prognosis in patients with acute leukemia and can have a major role as a parameter to guide risk-adapted therapy of this disease.

In our study, we analyzed 84 cases of acute myeloblastic leukemia (AML) and 16 cases of acute promyelocytic leukemia (APL). In these patients we quantified MRD by using a combination of monoclonal antibodies and polychromatic flow cytometry. We employed a FACSCanto II (Becton Dickinson). The different antigens were identified by six-color staining technique. The monitoring of MRD was effected, on bone marrow samples, after 15 days since the diagnosis and then every month. In each case we added to the conventional flow cytometry report a MRD progression chart in order to provide immediate information about the state and the course of the disease. We observed that the presence of a value of MRD of 0.1% (e. g. a cluster of 50 cells among 50,000 cells analyzed) was predictive of relapse in more than 90% of cases and it was a reason to perform more assiduous controls in those patients. In AMLs with CD34 negativity, asynchronous myeloid antigen (CD13 and CD33) expression or the presence of lineage infidelity, separately or at the same time, was a strong indicator of MRD. By contrast, coordinated expression of CD13+ and CD33+ on CD34+ cells testified, in general, a status of clinical remission.

The addition of a MRD progression chart to the conventional report seems to be a simple and useful instrument to monitor MRD. In our opinion the MRD progression chart, while providing an additional value to flow cytometry report, will contribute to understand the clinical significance of MRD as well as to modify therapeutic programs according to patient risk category.

PROTEIN KINASE C EPSILON (PKCε) AND ITS EMERGING ROLE IN HUMAN ERYTHRO/MEGAKARYOCYTOPOIESIS

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Protein kinase C (PKC)-mediated signalling participates in several key steps of hematopoietic cell differentiation. The ε isoform of PKC has been associated to erythroid (ER) differentiation as well as to the early phases of megakaryocytic (MK) lineage commitment. We worked on the hypothesis that PKCε expression levels might be modulated during ER and MK differentiation, with a specific role in the early as well as in the late phases of erythro/megakaryocytopoiesis. We demonstrate that EPO-induced CD34 cells are insensitive to the apoptogenic effect of TNF-related apoptosis-inducing ligand (TRAIL), a negative regulator of ER differentiation, at day 0 due to the lack of specific receptor expression. From day 3 onward, ER cells express death receptors and become sensitive to TRAIL up to day 7/8 when the EPO-driven up-regulation of PKCε renders ER cells resistant to TRAIL likely via Bcl-2 up-regulation. At variance with the ER lineage, PKCε is completely down-modulated in TPO-induced CD34 cells from day 6 onward. The forced expression of PKCε in the late phases of MK differentiation delays differentiation likely *via* Bcl-xL up-regulation. Moreover, TRAIL is not apoptogenic for TPO-induced CD34 cells, but rather accelerates their maturation. However, PKCε levels negatively interfere also with the differentiative effects of TRAIL. PKCε can therefore be considered a signalling intermediate whose expression levels are finely tuned, with a virtually opposite kinetic, in ER *vs* MK lineages, to adequately respond to the signaling requirements of the specific hematopoietic lineage.

DIAGNOSIS OF CHRONIC MYELOPROLIFERATIVE DISORDERS BY EIGHT COLOUR MULTIPARAMETRIC FLOW CYTOMETRY

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Chronic Myeloproliferative Disordes (CMD) are a group of clonal diseases characterized by deregulated proliferation and expansion of hematopoietic progenitors in bone marrow. Under this definition, different clinical, morphologic and biological disorders have been classified. To date, the assessment of these diseases is difficult, based upon morphologic criteria, with cytogenetic markers used as further classification support, because the proliferation of one or more of the myeloid lineages is often associated with normal maturation and the blasts percentage in the bone marrow is low. In this study we wish to demonstrate that it is possible to utilize efficiently immunological typing and flow cytometry criteria in order to obtain a rapid assessment of CMDs. In our laboratory, we used laser flow cytometer FACSCanto II equipped with three lasers (Becton Dickinson), by which we analyzed 30000 bone marrow cells per sample by 8-colour strategy. The panel of antibodies included CD71-FITC, CD14-PE, CD34-PerCP, CD10-PE-Cy7, CD33-APC, CD3-APC-Cy7, CD19-Pacific Blue, CD45-AMCyan, CD61-FITC, CD42b-PE. The combinations were the following:

A: CD71-FITC, CD14-PE, CD34-PerCP, CD10-PE-Cy7, CD33-APC, CD3-APC-Cy7, CD19-Pacific Blue, CD45 AMCyan;

B: CD61-FITC, CD42b-PE, CD34-PerCP, CD10-PE-Cy7, CD33-APC, CD3-APC-Cy7, CD19-Pacific Blue, CD45-AMCyan.

We propose 8-colour multidimensional flow cytometry as a suitable and easy method to depict all hematopoietic cells (erythroid cells, monocytes, blast cells, maturing granuloid cells, T and B lymphocytes, haematogones, platelets).

First of all, we studied 50 normal controls (patients affected by non-Hodgkin lymphoma without any bone marrow infiltration or patients with non hematological neoplastic disease not-affecting bone marrow). Then, we analyzed 10 patients with suspected CMDs. Among these 10 patients, we were able to diagnose 3 cases of essential thrombocythemia, 3 cases of primary polycythemia, 2 cases of chronic myeloid leukemia and 2 cases of chronic myelomonocytic leukemia. Our cytometric definition overlapped the clinico-hematological final diagnosis in 10 cases out 10, showing a complete agreement between flow cytometry and collective final assessment.

Thus, 8-color flow cytometry, at variance with what previously reported by the literature, appeared to be a useful tool to orientate the diagnosis of CMDs.

1073 GR/ML GMP-MANUFACTURED DENSITY GRADIENT IS RESCUING MESENCHYMAL STROMAL/STEM CELLS WITH A MORE POTENT IN VITRO PERFORMANCE

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Density gradient medium (DGM) at 1.077 g/ml is widely used to isolate mesenchymal stromal/stem cells (MSC) from bone marrow (BM). Since the protocol adopted in isolating of BM-MSC may influence the quality of adherent MSC populations, we hypothesized that the use of lower (1.073 g/ml) DGM may be associated with an enrichment of MSC progenitors having distinct physical and biological properties. Thus, we compared two novel GMP-manufactured DGM (General Electric Health Care) accordingly to their different densities (1.077 versus 1.073 g/ml).

BM samples (n=13) were separated by both GMP-DGM. The freshly isolated BM mononucleated cells (BMMNC) were tested for viability (by 7AAD), multiparametric flow cytometry analyses (CD45, CD14, HLA-DR, CD105, CD90, CD73, GD2, CD140, CD146, CD200), clonogenic MSC assay (CFU-F), MSC ex-vivo expansion and assessment of differentiation potentials.

No differences were noticed in cell viability between groups (7AAD+: 4,48±1,42% in 1.077 and 5,03±1,20% in 1.073). The FACS analyses on freshly isolated BMMNC indicate that 1.073 significantly reduces the CD45+ cell fraction and enriches CD45-/CD105+ sub-type in comparison with 1.077 GMP-DGM of approximately 1.25 fold. CFU-F and parameters dealing with cell expansion were significantly higher in the 1.073 group and, in particular, the average MSC yield was 1,5 fold more than 1.077. Both reagents could isolate MSC showing an expected phenotype however, 1.073-isolated MSC shown a higher percentage of cells expressing CD90, CD105, CD73 and GD2. Similarly, the mean fluorescence intensity reveals that cells isolated with 1.073 GMP-DGM shown higher CD90, GD2 and CD146 expression. Additionally, in both groups, MSC were capable to fully differentiate into bone, adipose cells and cartilage. In conclusion, our data suggest that 1.073 GMP-DGM provides a significant advantage in MSC isolation to be used into clinical trials.

CD34+ HUMAN PANCREATIC ISLET-DERIVED STEM CELLS DISPLAY ENDOCRINE/ENDOTHELIAL FEATURES AND MULTIDIFFERENTIATION POTENTIAL

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Stem cells offer exciting possibilities for the development of novel treatments for diabetes. Pancreatic islet-derived stem cells may bear advantages due to their tissue-specificity. We isolated, expanded and characterized stem cell populations from human pancreatic islets. Gentle isolation procedures were optimized for small pancreatic specimens. High yield expansion was obtained by culturing in Chang medium D. Adherent populations of fibroblast-like cells emerged in primary cultures and were extensively expanded. The cells were characterized by flow cytometry and immunofluorescence. Differentiation potential was investigated after induction with specific media. Highly expandable adherent populations were isolated. The cells expressed stem/progenitor markers (CD34+ Oct-4+ Sca-1+ SSEA-4+), displayed elevated expression of endocrine (Insulin+ Glucagon+) and endothelial/pericytic (vWF+ CD90+ CD105+ CD146+) markers. With the exception of CD34 positivity, the cells showed a phenotype similar to Mesenchymal Stem Cells (CD29+ CD44+ CD73+ CD90+ CD105+ CD166+ STRO-1+ CD14- CD45-). They showed multidifferentiation potential toward pancreatic endocrine and mesenchymal commitments. The cells had a considerable propensity to form islet-like clusters. Adherent fibroblast-like CD34+ stem cells can be isolated and expanded from human pancreatic islets: these cells display endocrine/endothelial features and remarkable multidifferentiation potential.

DIFFERENTIAL DIAGNOSIS BETWEEN MALIGNANT AND NORMAL PLASMACELLS BY MULTIPARAMETRIC FLOW CYTOMETRY

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The demonstration of the presence of phenotypically aberrant plasma cells can be used in the differential diagnosis between monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM). Plasma cells are characterized by high density of CD38 and CD138. Normal plasma cells do not express CD56 antigen, or do so at only a very low density. By contrast, CD56 is expressed very strongly on plasma cells in the majority of myeloma cases. CD19 and CD27 are positive in normal plasma cells, but they are, in general, negative in neoplastic plasma cells. CD28 is expressed only on some MM plasma cells. CD45 is absent on MM plasma cells and only lowly expressed on normal plasma cells.

In our study, we utilized 6-colour flow cytometry strategy using a FACSCanto II (Becton Dickinson) and we examined bone marrow samples from 60 patients, 32 with MM and 28 with MGUS. The number of total events acquired was consistently upper than 50,000. The following panel of monoclonal antibodies was used: CD38, CD138, CD56, CD19, CD27, CD28, CD9 and CD45. All 60 samples were positive for CD38 and CD138. The very strong expression of these antigens was used for gating strategy. In MGUS cases, only normal phenotype (CD19+/CD27+/CD56-/CD28-/CD45±) was present on 17/28 (60.7%) patients, while 11/28 cases (39.3%) showed the simultaneous presence of malignant (CD19-/CD27-/CD56+/CD28+/CD45-) and normal plasma cells. By contrast, in MM cases, 23/32 (71.8%) cases expressed only malignant phenotype and 9/32 (28.2%) showed the presence of both normal and malignant plasma cells. In addition, there was a direct correlation between fluorescence intensity of CD56, CD9 and CD28 on malignant plasma cells.

FLOW CYTOMETRY ANALYSYS OF EXTRAMEDULLARY MANIFESTATION IN MULTIPLE MYELOMA: REPORT OF 3 CASES

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Multiple myeloma (MM) is a haematological malignancy characterized by the occurrence of plasma cell tumours within the bone marrow. In advanced MM, metastatic deposits outside the bone marrow (extramedullary) are rare. Extramedullary plasmacytomas occur most frequently in the upper respiratory tract, lymph node, spleen, subcutaneous tissue, mediastinum. They may precede, be followed by, or be concurrent with MM. We describe 3 cases that reported extramedullary sites: 2 include the respiratory tract, 1 subcutaneous tissue. We utilized 6-colour flow cytometry strategy using a FACSCanto II (Becton Dickinson). 2 pleural effusions and 1 fine needle aspiration cytology (FNAC) were examined. The following panel of monoclonal antibodies was used: CD38 CD138 CD56 CD19 CD27 CD28 CD9 CD45. Plasma cells are characterized by high density of CD38 and CD138, the very strong expression of these antigens was used for a gate acquisition strategy. Neoplastic plasmacell infiltration was demonstrated. CD19 and CD27 were constantly negative. In all patients CD45 showed highly expression, in contrast with CD45 negative on bone marrow plasma cells in MM and only lowly expressed on normal plasma cells. In another study, CD45 bright has been shown to correlate with proliferating MM cells. In addition in our study, CD56 was negative (2/3 patients) or weakly positive (1/3). A strong association between the absence of CD56 expression and extramedullary spread has been described, possibly because high CD56 expression may restrict egress of tumor cells from the BM microenvironment.

MULTIPOTENT MESENCHYMAL STEM CELLS FROM AMNIOTIC FLUID ORIGINATE NEURAL PRECURSORS WITH FUNCTIONAL VOLTAGE-GATED SODIUM CHANNELS

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Objective. Amniotic fluid (AF) contains stem cells with high proliferative and differentiative potential which might be an attractive source of multipotent stem cells. We investigated whether human AF contains mesenchymal stem cells (MSCs) and evaluated their phenotypic characteristics and differentiation potential *in vitro*.

Methods. AF was harvested during routine prenatal amniocentesis at 14-16 weeks of pregnancy. AF sample pellets were plated in a-Mem with 10% Fetal Bovine Serum. We evaluated cellular growth, immunophenotype, stemness markers and differentiative potential during *in vitro* expansion. Neural Progenitor Maintenance Medium (NPMM, Lonza), a medium normally used for the growth and maintenance of neural stem cells, containing hFGF, hEGF, NSF-1 was used for neural induction.

Results. Twenty-seven AF samples were collected and primary cells, obtained from the samples containing more than 6 ml of AF, had MSC characteristics. AF MSCs showed a high proliferative potential, were positive for CD90, CD105, CD29, CD44, CD73, CD166, showed Oct-4 and Nanog molecular and protein expression and differentiated into osteoblasts, adypocytes and chondrocytes. The NPMM cultured cells expressed neural markers and increased Na+ channel density and channel inactivation rate making the TTX-sensitive channels more kinetically similar to native neuronal voltage-gated Na+ channels.

Conclusion. These data suggest that AF is an important multipotent stem cell source with a high proliferative

potential able to originate potential precursors of functional neurons.

MESENCHYMAL STEM CELLS EXPANSION BY PLATING WHOLE BONE MARROW AT LOW CELLULAR DENSITY: A MORE ADVANTAGEOUS METHOD FOR CLINICAL USE

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Mesenchymal stem cells(MSCs) are a prospective object for cell therapy. The identification of the "optimal" conditions is important to identify a standard procedure for clinical use. To this purpose, we used different in vitro MSCs separation and expansion methods. Percoll (1.073 g/ml), Ficoll (1.077 g/ml) and whole bone marrow directly plated without density-gradient were tested as separation methods and the cells were seeded in MSC Medium (Lonza) at the following densities: 100000, 10000, 1000, 100, 10 cells /cm^2. After reaching the confluence, the cells were detached, pooled and re-plated for further 3-5 passages at 1000, 500, 100 and 10 cells/cm^2. Statistical analysis were performed. The separation methods and seeding densities were not significantly different in terms of cumulative Poputation Doublings (PD), whereas the plating density showed significant differences of cumulative PD. Some small quantity samples plated in T25 or T75 flasks at plating densities of 10 and 100 cells/ cm^2 did not produce any expansion. Moreover, whole bone marrow directly plated resulted in a more advantageous method in terms of minimal manipulation and cellular growth (descriptive statistical analysis). No differences were observed in terms of gross morphology, differentiation potential and surface markers of cells isolated by the methods at different density.

All together these data suggest that whole bone marrow seeded at 100000 and plated at 1000 cells/cm^2 represents a good procedure for MSC expansion for clinical use compared to MSCs obtained by gradient separation.

TARGETING MYELOID MOLECULES TO KILL LYMPHOID CELLS: MESSAGES FROM A MULTIPLE PERSONALITY TUMOUR

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Lymphoplasmacytoid dendritic cell lymphoma (LP-DCL) is a rare and aggressive hematopoietic malignancy in which the response to conventional chemotherapy is poor. Malignant LP-DCL cells are featured by membrane expression of CD4 and CD56. During the last years, we characterized by flow cytometry (FCM) 8 patients with LP-DCL. Four of these were CD33+, in the absence of other myeloid markers. One of these CD33+ cases, an 18-year-old woman, was referred to our unit with LP-DCL at relapse phase. Here we want to show how the identification of CD33 expression in this case of LP-DCL had a key role for the therapeutic treatment. In December 2007, conventional cytology and FCM analysis evidenced cerebrospinal fluid infiltration of malignant cell characterized by CD4+/CD56+/TdT+/CD33+ immunophenotype.

Moreover, a BM cytometric study evidenced 20% infiltrating blasts expressing identical immunophenotype as compared to CSF. In January 2008, the patient was treated by DepoCyte intrathecal administration and systemic chemotherapy according to the Hyper-CVAD scheme. Following the first two DepoCyte administrations, FCM showed complete CSF infiltration clearance. In February, based upon CD33 positivity, systemic gemtuzumabozogamicin (G-O) administration was introduced in order to eradicate chemotherapy resistant LP-DCL cells. In March, the patient started Hyper-CVAD second block and one month later, a FCM myeloaspirate inspection documented BM minimal residual disease (MRD) at 0.1% level. After a second G-O dose, BM MRD was 0.045% and, following an additional infusion (in May), the BM MRD was finally undetectable.

In September, after autologous BM transplantation, she received a fourth G-O infusion and no cancer cells were detectable by FCM. In October, the patient was well; she received a fifth G-O administration and to date BM MRD is negative. SCHEDA ABSTRACT

FLOW CYTOMETRY ANALYSIS OF B-CELL RECEPTOR PHOSPHOPROTEIN NETWORKS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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B-cell chronic lymphocytic leukemia (B-CLL) patients exhibit a variable clinical course. Several biological parameters have been shown to be associated with clinical outcome in CLL. Among them, the most reliable markers are represented by the absence of somatic mutations within the immunoglobulin variable heavy chain genes (*IGHV*), the expression of CD38 antigen, the presence of the ZAP-70 tyrosine kinase. These parameters of poor clinical outcome are structurally and/or functionally linked to B-cell Receptor (BCR) expressed by CLL cells, thereby strengthen the hypothesis that antigenic stimulation mediated by the BCR represents a driving event in the onset and progression of the malignant B cells.

To investigate whether different BCR signaling networks may distinguish clinical-biological groups of CLL patients, we applied a "network level" view of BCR signaling by analyzing single-cell profiles of phospho-protein networks by flow cytometry. We evaluated the response to BCR engagement in primary cells isolated from 27 CLL patients by analyzing the phosphorylation states of 5 phospho-proteins on the route of BCR signaling, which included p-Syk, p-NF-kappaB, p-Erk1/2, p-p38 and p-JNK. BCR was cross-linked by incubating cells with anti-IgM antibodies. The unsupervised clustering analysis distinguished BCR response profiles of phospho-proteins that differentiated cases of CLL with mutated *IGHV* from those with unmutated *IGHV* ($p = 0.0003$), cases with low levels of CD38 from those with high levels ($p = 0.0004$) and cases with low levels of ZAP-70 from those with high levels ($p = 0.001$). Furthermore, the same BCR response profiles were also associated with time to progression ($p = 0,0014$) and with overall survival ($p = 0.049$), as assessed by Kaplan–Meier curves and the log-rank test.

This study shows that single-cell profiles of BCR phospho-protein networks are associated with prognostic parameters and disease progression in CLL.

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DIAGNOSTIC UTILITY OF FLOW CYTOMETRY IN LOW-GRADE MYELODYSPLASTIC SYNDROMES: A PROSPECTIVE VALIDATION STUDY

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Background: The diagnosis of myelodysplastic syndromes is not always straightforward when patients lack specific diagnostic markers, such as blast excess, karyotype abnormality, and ringed sideroblasts.

Design and Methods: We designed a flow cytometry protocol applicable in many laboratories and verified its diagnostic utility in patients without those diagnostic markers. The cardinal parameters, analyzable from one cell aliquot, were myeloblasts (%), B-cell progenitors (%), myeloblast CD45 expression, and channel number of side scatter where the maximum number of granulocytes occurs. The adjunctive parameters were CD11b, CD15, and CD56 expression (%) on myeloblasts. Marrow samples from 63 control patients with cytopenia and 88 low-grade myelodysplastic syndromes patients, including 55 lacking both ringed sideroblasts and cytogenetic aberrations, were prospectively analyzed.

Results: Data outside the predetermined reference range in 2 or more parameters (multiple abnormalities) were common in myelodysplastic syndromes patients. In those lacking ringed sideroblasts and cytogenetic aberrations, multiple abnormalities were observed in 37/55 patients (67.3%) when the cardinal parameters alone were considered, and in 42/47 patients (89.4%) when all parameters were taken into account. Multiple abnormalities were rare in controls. When data from all parameters were used, the diagnostic sensitivities was 89%, specificities was 90%, and likelihood ratios was 8.5.

Conclusions: This protocol can be used in the diagnostic work-up of low-grade myelodysplastic syndromes patients who lack specific diagnostic markers, although further improvement in diagnostic power is desirable.

MOBILIZATION OF MESENCHYMAL STEM CELLS IN PATIENTS WITH CONGESTIVE HEART FAILURE

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Background: Heart failure is one of the most common causes of death in the world. Currently, cellular replacement therapy has emerged as a novel strategy for the treatment of heart failure.This possibility, called cardiomioplasty, consists in transplantation of stem cells able to restore the normal cardiac functions. Mesenchymal stem cells (MSCs) represent the optimal candidate for heart regeneration: they are quite easy to isolate, have high expansion potential and are able to differentiate in cardiomyocytes.

In a previous study, our group demonstrated that CD34+ hematopoietic stem cells increase in patients at the early phases of HF and decrease in more severe patients, i.e. NYHA III and IV. Starting from these data, we aimed to assess the levels of circulating mesenchymal cells in patients with HF and correlate them with severity of the disease.

Methods: Quantification of MSCs in peripheral blood of patients affected by chronic heart failure was performed with specific monoclonal antibodies on Beckman Coulter CyAn flow cytometer. The role of the pathological enviroment on MSCs mobilization was assessed by specific biomarkers titration by ELISA.

Results and Conclusions: Our data confirm the relationship between MSCs mobilization and HF-stage. In particular, the level of circulating MSCs appears inversely related to the severity of the desease, indicating a possible role of MSCs in restoring the cardiac function only in the first phases of the pathology, while in severe HF patients MSCs action seem to be not effective. Other studies are necessary to investigate cellular and/or molecular mechanisms related to this phenomenon.

Study supported from Emilia Romagna Region (Project: Establishment of a regional network to investigate, by applying a translational approach, the role of stem cell therapy in Coronary Artery Disease (CAD) patients with advanced left ventricular (LV) dysfunction. Area: Regenerative Medicine).

CYTOMETRIC EVALUATION OF MUTATED CLONES IN A LARGE COHORT OF PNH PATIENTS

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired disease characterized by intravascular hemolysis, bone marrow failure, hemoglobinuria and increased thrombotic events. It is determined by a deficiency of GPI-anchored proteins on hematopoietic cells. Flow cytometry is considered the best tool for PNH clone detection. Several protocols have been proposed, including (1) CD59 as unique reagent to screen all hematopoietic lineages and (2) multiparametric combinations (e.g. CD24/CD16/CD45) to analyze granulocytes. In this study, we used CD66b for granulocytes, CD14 for monocytes, CD59 for erythrocytes, CD48 for lymphocytes and FLAER for leukocytes. Antibody combinations were the following:

1. CD66b/CD33/CD45; 2. CD14/CD33/CD45; 3. CD48/CD3/CD45; 4. CD48/CD19/CD45; 5. CD48/CD56/CD45; 6. FLAER/CD33/CD45; 7. CD59 (as mono-parametric staining for RBC). Data of 36 patients diagnosed as PNH in the past 2 year were collected.

Results (expressed as % of cells negative for the indicated GPI-linked molecules).

	$CD66b^{neg}$ grans	$FLAER^{neg}$ grans	$CD14^{neg}$ monos	$FLAER^{neg}$ monos	$CD59^{neg}$ RBC	$CD48^{neg}$ T cells	$CD48^{neg}$ B cells	$CD48^{neg}$ NK cells
Min	0.3	15	4	28	0.3	0	0	0
25%	49	49	54	55	8		0	1
Med	84	78	87	89	28	4	5	7
75%	49	49	54	55	8	0	0	1
Max	99	99	100	99	99	44	91	95

As shown, the patient cohort included cases with minimal PNH clones ("subclinical PNH") accompanied by cases in which PNH clone represents the sole hematopoietic resource ("florid PNH" in absence of polyclonal hematopoiesis). As regards the use of FLAER, its correlation with GPI-linked antigens was absolute: CD66b vs FLAER: r=1.0, p=0.0004; CD14 vs FLAER: r=1.0, p=0.0004. This study indicates (i) the reliability of our diagnostic protocol, (ii) the heterogeneity of PNH patients in terms of width of PNH clone; (iii) the absolute overlapping between FLAER and GPI-linked molecule detection on granulocytes and monocytes.

FLOW CYTOMETRY LOCALIZATION OF PML AS A TOOL FOR DIAGNOSIS AND EVALUATION OF MINIMAL RESIDUAL DISEASE IN ACUTE PROMYELOCYTIC LEUKEMIA

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In acute promyelocytic leukemia (APL) rapid diagnosis allows early administration of all-trans retinoic acid (ATRA), which ameliorates the severe coagulopathy. PCR detection of PML/RARα, the gold standard in APL diagnosis, is prone to contamination and must be carried out in experienced laboratories. On the other hand, flow cytometry is also sensitive and specific, but good results are possible only in very skilful hands. A recently developed technique, the PML immunofluorescence, simple but entirely manual, is not yet universally used to rapidly provide a diagnosis of APL.

ImageStream is a multispectral imaging flow cytometer. The platform produces high resolution bright-field, dark-field and fluorescence images of cells prepared in suspension. The IDEAS software quantifies over 500 morphmetric and photometric parameters for each cell based on its imagery, including parameters that measure sub-cellular location of probes. This technology combines the quantitative power of cytometry with the high information content present in microscopic images. The localization of PML in NB4 and HL60 was measured using image-based analysis on the ImageStream. About 20,000 cells per sample were collected, which enabled quantitative image based analysis of PML localization. In addition, NB4 cell detection in the midst of HL60 cells was also studied using electronic data mixing of various ratios of NB4 and HL60 events in the files. Using the Spot Count and Modulation features, we quantified the localization of PML within NB4 (APL) and HL60 (non-APL) cells. We observed more spots per cell and higher fluorescence modulation in HL60 cells than in NB4 cells. We propose the electronic detection of PML localization as a new tool to rapidly diagnose APL as well as to detect minimal residual disease in this peculiar type of acute leukemia.

ANALYSIS OF SEROUS EFFUSIONS AND BRONCHOALVEOLAR LAVAGES FROM PATIENTS WITH HEMATOLOGIC NEOPLASM: COMPARISON OF FLOW CYTOMETRY AND CYTOMORPHOLOGY WITH RETROSPECTIVE CLINICAL ASSESSMENT IN 84 CASES

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The differential diagnostic potential of flow cytometry (FC) and cytomorphology (CM) in the analysis of (i) body cavity fluids (BCF) according to different diagnostic settings and (ii) bronchoalveolar lavages (BAL) from patients with hematologic neoplasm (HN) is largely undetermined. We selected BCF analyzed by FC with (i) suspected or known HN at the time of withdrawal, (ii) CM performed on the same sample, and (iii) availability of follow-up findings for retrospective clinical assessment (RCA). FC and CM results

were compared to RCA. Inter- and intra-method comparisons were performed by means of ROC curve analysis and Chi Square test, respectively.

Eighty-four samples, 57 serous effusions (SE) and 27 BAL, submitted for suspected (38%) or disclosed (staging: 11%, suspect of relapse/progression: 51%) HN were selected for analysis. During a median follow-up of 4 months, 24 SE and 8 BAL were found positive by RCA. Overall, 100% specificity was detected for both methods; FC retained significantly higher sensitivity as compared to CM (87.5% vs 46.2%, p = .0006). The highest FC accuracy (100% sensitivity, 100% NPV) was displayed in the analysis of T-cell precursor non Hodgkin lymphoma (NHL)/leukemia and of T-cell differentiated NHL. FC was significantly more sensitive than CM in the subsets of B-cell differentiated NHL (85.7% vs 37.5%, p = .0006) and B-cell precursor NHL/leukemia (86.2% vs 41.7%, p = .0005). Similarly, FC displayed a better diagnostic value than CM in the analysis of samples submitted in the suspect of relapse/progression (sensitivity 90% vs 41.2%, p = .0002). Although FC accuracy in the BAL setting was lower than that displayed in the SE setting (sensitivity 75% vs 91.7%, p = .08), immunophenotyping detected neoplastic cells in 6 out of 8 samples from patients affected by B-cell (n = 4) or T-cell (n = 4) differentiated NHL, whereas CM gave 100% false negative results.

FC is the best diagnostic tool for detecting neoplastic cells in BCF from patients with T-cell lineage NHL/leukemia; a stricking diagnostic advantage is suggested for FC in the analysis of BAL.

CEREBROSPINAL FLUID EXAMINATION IN 123 CASES OF HEMATOLOGIC MALIGNANCY: FLOW CYTOMETRY ACCURACY DEPENDS ON THE NUMBER OF ACQUIRED CD45$^+$ CELL EVENTS

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In active leptomeningeal hematologic malignancy (HM), flow cytometry (FC) results on serial cerebrospinal fluid (CSF) samples are a decision making criterion for managing intrathecal treatment (ITT).

We selected CSF analyzed by FC with (i) suspected or known HM at the time of withdrawal, (ii) cytomorphology (CM) performed on the same sample, and (iii) availability of follow-up findings for retrospective clinical assessment (RCA). FC and CM results [positive (for FC, at least 15 events consistent with HM phenotype) or negative for neoplastic cells] were compared to RCA. Inter-method comparisons were performed by means of ROC curve analysis.

One hundred twenty-three CSF submitted for suspected (3%) or disclosed HM {21% prior to treatment [differentiated non Hodgkin lymphomas (NHL)], and 76% during follow-up [44 differentiated NHL, 36 precursor NHL/leukemias, 13 acute myeloid leukemias]} were selected for analysis. Overall, 100% specificity was detected for both FC and CM; as compared to CM, FC retained significantly higher sensitivity (74.3% vs 63.3%) and negative predictive value (90.2% vs 87.8%)(p = .014). FC displayed a better diagnostic value than CM in the analysis of samples submitted prior to any ITT (sensitivity 90% vs 62.5%, p = .127) and after at least one ITT (sensitivity 63.6% vs 57.9%, p = .061). When acquisition cut-offs were tested, 100% sensitivity was observed for FC by acquiring at least 50 total CD45$^+$ cell events, the correspondent sensitivity by CM being 75%. When fewer events could be acquired, the best sensitivity was instead obtained with CM by considering positive also uncertain results (CM-U$^+$): it was higher than that of FC when either < 20 (50% vs 0%) or 20-50 (75% vs 50%) total CD45$^+$ cell events were acquired by FC.

FC seems to be the most accurate method when at least 50 CD45$^+$ cell events can be acquired; in the other cases CM-U$^+$ retains diagnostic advantage. In particular, the 20 events cutoff should be considered to distinguish true negative results from "quantity not sufficient" for FC analysis.

BASAL CD34+ CELL COUNT AS PREDICTOR FACTOR OF PERIPHERAL BLOOD PROGENITOR CELL MOBILIZATION AND COLLECTION IN HEALTHY DONORS AFTER ADMINISTRATION OF LENOGRASTIM

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No specific characteristics have been identified as predictors of hematopoietic progenitor cells (HPC) mobilization in healthy donors. Literatures' data showed that some donors are poor responders to recombinant granulocyte colony-stimulating factor (rhG-CSF) and that the baseline number of CD34(+) cells correlates with the number of CD34(+) cells in the peripheral blood (PB) at the day of apheresis. Thus, the number of CD34+ cells circulating in PB at steady state can be used as useful indicator of CD34(+) cell mobilization after rhG-CSF administration. The purpose of this study was trying to identify clinically significant factors that could influence the effectiveness of CD34+ cell mobilization and collection with special focus on the value of the basal CD34+ cells. 24 healthy donors underwent to apheresis procedures on day 5 of treatment with rhG-CSF were prospectively analyzed for correlations with CD34(+) cell mobilization. The variable was examined separately by linear regression analysis against independent variables (sex, age, weight, dose of rhG-CSF, baseline white cell count, and baseline CD34+ cell count). By univariate analysis, male sex (P = 0.007), body weight (≤70 vs. >70 kg, P = 0.04), and donor's age (≤50 vs. > 50 years; P = 0.015) were correlated with the number of CD34(+) cells mobilized but not with basal CD34+ value (P = n.s.). By multivariate analysis, donor's age and male sex were the only two variables that significantly predicted an high CD34(+) cell level. In conclusion, our data suggest that male sex and younger age are the only factors that significantly affect CD34(+) mobilization in healthy donors.

IMPACT ON THE IMMUNOLOGICAL RECONSTITUTION OF A GRAFT CONTAINING MORE THAN 5x10E6/Kg CD34+ CELLS

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It is know that early recovery of absolute lymphocyte count (ALC≥500 cells/μL at day 15) after autologous peripheral blood haematopoietic stem cell transplantation (ASCT) represents a powerful prognostic indicator of clinical outcome, being correlated with better values of Overall Survival (OS) and Progression Free Survival (PFS). In a setting of 144 patients with Non Hodgkin's Lymphoma underwent to ASCT, who have received a median dose of CD34+ cells equal to 4.8 × 10e6/kg (r. 2.0-16.0), we have demonstrated a remarkable impact of ALC-15 on OS (0.0051) and PFS (0.0026). Therefore, we prospectively assessed whether increasing the number of infused CD34+ cells, it was possible to obtain a more rapid and stable immunological reconstitution and a better clinical outcome. We evaluated 27 pts, with different diagnosis (14 with lymphoma, 10 with myeloma and 2 with acute leukemia), underwent to ASCT and that have received more than 5×10E6/Kg CD34+ cells, mean 6.6 (r.5.9-7.7). At day 15 the immunophenotyping was performed using a BD FacsCalibur to evaluate the reconstitution of the subsets T, B and NK. We observed that the median number of ALC in this patients was 397 /μL and in particular in only ten pts we observed an ALC > 500/μl, suggesting no correlation between CD34+ peripheral blood progenitor cell dose and early lymphocyte recovery. Based on these data, we can hypothesize that a threshold number of CD34+ cells should not be the only parameter considered for an adequate PBSC collection and could be needed new mobilizing drugs to improve the quality of the graft.

FLOW CYTOMETRIC ANALYSIS OF PROGENITOR AND CIRCULATING ENDOTHELIAL CELLS IN TYPE 2 DIABETES

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Endothelial Progenitor Cells (EPCs) are the major factor promoting angiogenesis during adult life and represent extremely rare events in peripheral blood (from 0,01% to 0,001% of MNC). Circulating Endothelial Cells (CECs) are detached from endothelium in response to pathological insults. EPCs and CECs could be considered as markers of endothelial health and damage, respectively. Type 2 Diabetes (T2D) is a multifactorial disease that leads to endothelial dysfunction. Therefore, measurements of changes in EPCs and CECs could be a useful tool in diagnosis or prognosis of endothelial dysfunction.We analyzed different subsets of EPCs and CECs in healthy volunteers (n = 27), diabetic patients with no clinical evidence of angiopathy (n = 27) and diabetics with peripheral arterial occlusive disease (PAD) (n = 27), using a high performance flow cytometer BD FACSCanto. We identified living pre-EPCs (CD117/CD34/CD133), EPCs (CD34/CD133/VEGFR2), late-EPCs (CD31/VEGFR2/Ve-Cadherin), living and dead CECs (CD146/CD31/CD45neg). We found that T2D induced a significant decrement of late-EPCs, whereas EPCs was not significantly decreased. T2D patients (but not T2D-PAD patients) showed higher numbers of pre-EPCs than healthy people. The endothelial damage induced by T2D was confirmed by the increment of CECs. Our results suggest that: i) T2D-induced endothelial damage is attributable more to an altered process of maturation of EPCs than to a simple decrease in their production; ii) CECs could be a useful marker for assess the severity of endothelial damage.

IMMUNOLOGY

EIGHT COLOURS FLOW CYTOMETRIC ANALYSIS OF DENDRITIC CELLS SUBSETS IN MOUSE LYMPHOID ORGANS

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Dendritic cells (DC) are critical decision-making cells involved in immunity. They direct key functions including tolerance, anergy and initiation of adaptive immune responses. The murine DC family comprises heterogeneous cell populations with different phenotype, localization and response to activating stimuli. However, most of the antigens closely associated with DC cells are not functionally characterized. Moreover, some of the monoclonal antibodies routinely used are orphans and the recognized antigen has not been defined. To better approach the study of DC subpopulations we propose a eight colours flow cytometric analysis of murine DCs isolated from secondary lymphoid organs, including spleen, mesenteric and inguinal lymph nodes. Using a combination of the following antibodies: CD11c, CD11b, B220, PDCA-1, Siglec-H, CD4, CD8α, DEC205, CD80, CD86, MHC-II, CD16/CD32, we identified cell populations with different patterns of surface molecules among the classical DC subsets.

This method represents an up to date approach for the identification of DC subpopulations likely to play an important role in the activation of nonoverlapping repertoires of $CD4^+$ T cells.

PHENOTYPE AND FUNCTION OF THYMIC CD4SPCD25+ CELLS IN MYASTHENIA GRAVIS

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Myasthenia gravis (MG) is often associated with thymoma (Thy) or thymic follicular hyperplasia (TFH), and intrathymic T regulatory cells (Treg) seemingly play a pathogenic role. We examined 9 MG-Thy, 11 TFH and 5 non MG-Thy in comparison with the available literature data on human young thymus. We found a normal Treg ($CD4SPCD25^+$) frequency, although a low Treg proportion in TFH expressed the differentiation markers CD45RA and CD69. Treg frequency was reduced in MG- and non MG-Thy, but $CD45RA^+CD69^+$ Treg frequency was close to normal value in the former, in line with the view that tumourous changes in MG-Thy are like a 'second childhood' in the thymus. In all thymuses, $CCR4^+$Treg frequency was high suggesting a hampered Treg export. Treg (immunomagnetically purified as $CD4SPCD25^+$ cells) in TFH were fully functional, whereas Treg in both MG- and non MG-Thy had an inconsistent suppressive activity with no relationship with Foxp3 expression indicating that even the presence of this pivotal marker does not necessarily equate to a bona fide Treg. We then looked at the effects of prednisone on Treg (4 MG-Thy and 6 TFH). Thymuses were stratified according to steroid sensitivity defined as the extent of DP thymocyte depletion. Treg frequency slightly increased in steroid-sensitive thymuses, and Treg were more differentiated, as judged by the enhanced CD45RA expression. However, CCR4 expression also increased, suggesting that steroid-induced Treg had a scarce propensity to leave the thymus, in line with our earlier data showing that the thymic contribution to peripheral Treg pool in MG is dispensable.

IMMUNOLOGICAL EVALUATION OF SUBJECTS WITH CRI DU CHAT SYNDROME (5P-)

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The Cri du Chat syndrome (CdCS) is a genetic disease resulting from a deletion of variable size occurring on the short arm of chromosome 5 (5p-). The incidence ranges from 1:15,000 to 1:50,000 live-born infants. The main clinical features are a high-pitched monochromatic cry, some facial and body dysmorphysms and severe psychomotor and mental retardation. As for other genetic syndrome (i.e. Down syndrome) CdCS subjects are told to be prone to infections, as a result of immune deficiency.

We studied 22 subjects with CdCS, 11 females and 11 males, 5 – 32 yrs old. They were evaluated by physical examination and medical history; complete blood count; serum immunoglobulin (IgG, IgA, and IgM) levels; C3 and C4 levels; and lymphocyte subsets.

Results: no subject had a personal history suggestive of possible immune deficiency. A mild anaemia was present in male subjects (Hb12.9 +/− 2.9 g/dl). The levels of C' were normal, while most of subjects showed increased values of IgG and IgA. A complex derangement of the major peripheral blood cell subsets was observed, with different characteristics in males and females, in comparison to the control group. In males, a significant decrease of the absolute number of circulating lymphocytes was present, due to the reduction of CD3+CD4+ cells (681 +/− 195 vs 1125 +/− 301). Females showed a different distribution, with a significant decrease of CD8+ cells in percentage but not in absolute number, increased CD4/CD8 ratio (2.4 +/−1 vs 1.5 +/−1) and increased percentage of B (CD20+) lymphocytes (11.6 +/−5.4 vs 8.5 +/− 3.4). Finally some patients showed increased values of CD19+CD5+ cells, with discrepancies between CD19+ and CD20+ cells, without evidence of haematologic or autoimmune diseases.

PHENOTYPYC ANALYSIS OF OVINE PERIPHERAL BLOOD AND MILK LYMPHOCYTES DURING LACTATION

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Somatic cell count (SSC) is considered a well established indicator of udder health status in lactating sheep. However, few information are still available on composition of lymphocytes subsets in sheep mammary gland and its secretion. The present investigation was undertaken to evaluate in flow cytometry ovine milk and blood lymphocytes subsets (CD4, CD8, WC1, CD25) throughout lactation. Samples were obtained from adult sheep (n=50) at early, middle and late lactation stage. Our results revealed that ovine milk contains CD4+ (helper/inducer), CD8+ (suppressor/cytotoxic) and WC1+ δγ T cell subsets which undergo changes during different lactation periods. These variations appeared evident in late lactation when it could be noticed a CD8+ decrease ($P<0,01$) in milk and CD4+ increase both in blood and milk. CD4+ lymphocytes showed a larger CD25 coexpression than CD8+ cells in blood as well as in milk, especially evident in late lactation. A comparison between lymphocytes subsets in blood and mammary compartment respectively evidenced lower CD8+ (15,8±4,1% *vs* 61±19%) and higher CD4+ (29,6±7,1% *vs* 14,1±8,7%) proportion at all time points, as evidenced by the CD4/CD8 ratio inversion (2,04±0, *vs* 0,3±0,3). Lower WC1+ percentages were also found in blood respect to milk (9±4,9 *vs* 34,5±14,2). Further phenotypical and functional studies on milk lymphocytes subsets would be helpful to gain a better understanding on mammary gland immune response against mastitis agents.

$CD4^+CD25^+FOXP3^+$ NATURAL T REGUALTORY CELLS SELECTION

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$CD4^+/CD25^+$ T regulatory cells (Tregs) are a potentially powerful tool in bone marrow transplantation. We

isolated Tregs from standard leukapheresis products using double-negative selection (anti-CD8 and anti-CD19 monoclonal antibodies) followed by positive selection (anti-CD25 monoclonal antibody). The final cell fraction ($CD4^+/CD25^+$) showed a mean purity of 93.6% ±1.1. Recovery efficiency was 81.52%±7.4. The $CD4^+/CD25^{+bright}$ cells were 28.4% ±6.8. The $CD4^+/CD25^+$ fraction contained a mean of 51.9%±15.1 FoxP3 cells and a mean of 18.9%±11.5 CD127 cells. The distribution of CD45 isoforms within CD4+/CD25+ fraction was 95.3±2.3 for CD45RO and 5.3%±0.25 for CD45RA. CCR5 and CCR7 constituted respectively 21%±14.8 and 0.5%±0.3 of the CD4/CD25+final fraction. CD62L+ cells were 79.5±6.1. The inhibition assay showed $CD4^+/CD25^+$ cells inhibited $CD4^+/CD25^-$ cells in a dose-dependent manner (mean inhibition percentages: 72.4± 8.9 (ratio Tresp/Tregs 1:2); 60.8± 20.5%. (ratio Tresp/Tregs 1:1); 25.6±19.6 (ratio Tresp/Tregs 1:0.1). Our study shows negative/positive Treg selection, significantly enriches $CD4^+CD25^+FoxP3^+$ cells endowed with immunosuppressive capacities. The $CD4^+CD25^+FoxP3^+$ population is a source of natural Treg cells that are depleted of $CD8^+$ and $CD4^+/CD25^-$ reacting clones which are potentially responsible for triggering Graft versus Host Disease (GvHD).

IS HLA-DRB4 * PREDICTIVE FOR A VASCULITIC SYNDROME IN ASTHMATIC PATIENTS? PRELIMINARY REPORT

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Introduction. The Churg-Strauss syndrome is a rare eosinophil-rich systemic necrotizing vasculitis characterized by severe asthma, in which HLA-DRB4 * is present in 65% of patients. Aim of this study was to evaluate retrospectively the prevalence of HLA-DRB4 * in a cohort of patients with persistent asthma and its possible clinical significance. Patients and methods. We calculate the HLA-DRB4 gene frequency by summing up the frequencies of the HLA-DRB1 *04, *07 and *09 alleles (strong linkage disequilibrium). We correlated the presence of HLA-DRB4 * with the clinical and laboratory features in 158 unselected patients with history of various degrees of persistent asthma. Statistical analysis was performed. Results. HLA-DRB4 * is present in 59 of 158 patients (37.3%). In HLA-DRB4* positive patients were higher: 1) the number of patients needing high dose of inhaled steroids to achieve asthma control: 23 of 59 vs. 15 of 99 ((39% vs. 15.2%, $p = 0.0007$); 2) the number of patients with at least one emergency admission for severe hypoxemic or near fatal asthma: 13 of 59 vs. 7 of 99 ((22% vs. 7.1%, $p = 0.006$); 3) the number of patients needing daily oral steroids to achieve asthma control: 14 of 59 vs.5 of 99 (23.7% vs. 5.1%, $p = 0.00048$); 4) the number of patients with eosinophils > 1500 cell/mm^3: 9 of 59 vs. 3 of 99 (15.3% vs. 3%, $p = 0.005$). Conclusion. HLA-DRB4* in asthmatic patients seems to distinguish a more severe clinical pattern in which eosinophilic inflammation and the severity of asthma attacks are prevalent. This clinical form resembles to the asthmatic prodromal phase of Churg-Strauss vasculitis: HLA-DRB4 * may be helpful to predict Churg-Strauss syndrome before the vasculitic phase in patients with persistent asthma.

ROLE OF THE CHEMOKINE DECOY RECEPTOR D6 IN ADAPTIVE IMMUNE RESPONSES

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The atypical chemokine receptor, D6, binds a broad range of pro-inflammatory CCchemokines but lacks sequence motifs that are required for the G-protein coupling and signalling functions of chemokine receptors. It is expressed mainly by lymphatic endothelial cells and placental trophoblasts, although expression on hematopoietic cells has also been reported. The receptor has been shown to act as a scavenging or decoy receptor. Indeed, mice deficient for D6 are developmentally normal but display exaggerated cutaneous inflammatory pathology upon phorbol ester application or injection of Freund's complete adjuvant. However, the precise role of D6 in adaptive immune responses has not been addressed. In one report, D6-deficient mice were shown to be unexpectedly more resistant to EAE, and although deficient DC migration to the immunization sites was proposed as an explanation, the activation and proliferation of antigen-specific T cells was not addressed.

The initiation of efficient adaptive immune response involves the arrival and encounter of dendritic cells and T cells within secondary lymphoid organs.

Our goal in this study was to determine whether D6 plays a role in the initiation of adaptive immune responses. For this, we have carefully examined the migration capacity of bone marrow derived D6-/- and wild-type dendritic cells into draining lymph nodes as well as their capacity to prime antigen-specific T cell responses in vivo using flow Cytometer technique.

IDENTIFICATION OF CELLULAR MECHANISM INDUCED BY ADVERSE DRUG REACTIONS TO ACETYLSALICYLIC ACID THROUGH FLOW CYTOMETRY ASSESSMENT OF CD63 AND CD203C

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Primary action of Acetylsalicylic Acid (ASA), is the inhibition of cyclooxygenases that are involved in Arachidonic Acid (AA) conversion to prostanoids. Often ASA induces adverse reactions (ADR). Despite its risks the use of

ASA is much diffused in the world. In addition it is important to remember that salicylates are content in different vegetables and fruits.

A rational approach to study the ADR mechanisms is to investigate on several reaction pathways for one molecule at a time. For this we studied the reactions induced by ASA on basophils from hypersensitive subjects, by assessment of two different markers: CD63 and CD203c, well correlated to allergic reaction. CD63 evidences degranulation process by preformed inflammatory mediators release. CD203c seems to be correlated to activation of AA degradation pathways that lead to the production of further mediators.

To perform this study were selected 230 subjects (115 healthy and 115 allergic to ASA). For each sample a negative control was prepared to establish Patient Background (PB) and 2 positive controls. Each one was also stimulated by ASA. The labelled samples were evaluated by flow-cytometry. A positive threshold was calculated by Stimulation Index (SI), [$CD63_{ASA}/CD63_{PB}$] and [$CD203c_{ASA}/CD203c_{PB}$] ($SI \geq 2$).

Healthy subjects did not show any positivity to both tests, while among allergic subjects we observed 105 on 115 subjects positive to at least one of the tests (91,30%), and 10 negative subjects for both tests (8,70%). In particular 78% of subjects were positive for CD63 expression, and a 30% of them were CD203c positive. The 14% of our patients was double-positive. From these observation we could suppose that in allergic subjects, ASA preferentially enhances degranulation process. In addition we conclude that the association of these two tests is very reliable in diagnosing ASA hypersensitivity.

HUMAN CYTOMEGALOVIRUS-SPECIFIC AND γδ T-CELLS IN CHILDREN RECEIVING HEMATOPOIETIC STEM CELL TRANSPLANT

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Background: Expansion of circulating $V\delta2^-$ γδ T-cells was observed in organ recipients in response to human cytomegalovirus (HCMV) infection. We investigated γδ T-cells and HCMV-specific $CD4^+$ and $CD8^+$ T-cells in children receiving hematopoietic stem cell transplant (HSCT).

Methods: $V\delta2^-$ and $V\delta2^+$ γδ, and HCMV-specific T-cells were determined by high resolution flow cytometry (FC500 Beckman Coulter equipment) in 7 children during the first year after HSCT. HCMV-infected autologous dendritic cells were used as a stimulus to detect specific IFN-γ producing $CD4^+$ and $CD8^+$ T cells.

Results: HCMV was detected in blood of 6/7 patients. In 4 patients (pts #1-4) HCMV infection developed within two months and was cleared between 76 and 197 days after HSCT. Expansion of $V\delta2^-$ γδ T-cells was observed between 49 and 92 days after HSCT, following a kinetics similar to HCMV-specific $CD8^+$ T cells. Specific $CD4^+$ T-cell appearance was delayed. One patient (pt #5) with a delayed-onset and sustained HCMV infection (from day 122 to day 391) showed a delayed development of HCMV-specific $CD8^+$ and γδ T-cells (>180 days) with sustained lack of specific $CD4^+$ T-cells. Early simultaneous development of $CD4^+$ and $CD8^+$ HCMV-specific T-cell response but not increase in $V\delta2^-$ γδ T-cells was observed in another patient (pt #6) with a short and early episode of infection. Finally, neither development of $CD4^+$ and $CD8^+$ HCMV-specific T-cell response nor $V\delta2^-$ γδ T-cell expansion was observed in a patient (pt #7) who did not experience HCMV infection. No expansion of $V\delta2^+$ γδ circulating T-cells was observed in any of the 7 patients studied.

Conclusions: The expansion of circulating $V\delta2^-$ γδ T-cells appears to correlate with HCMV infection in children receiving HSCT. The relationship between $V\delta2^-$ γδ T-cells and HCMV-specific $CD4^+$ and $CD8^+$ T-cells and their role in the control of HCMV infection should be further investigated.

SOLUBLE HLA CLASS I / CD8 LEGATION TRIGGERS TGF-β1 MOLECULES SECRETION IN ACTIVATED T LYMPHOCYTES AND NK CELLS

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The mechanisms involved in maintaining lymphocyte homeostasis are poorly understood. These cooperative interactions involve numerous cytokines acting through specific membrane receptors. To this topic, we have shown that the binding of soluble HLA class I molecules to CD8 on activated T and NK $CD8^+$ cells, induces up-regulation of Fas-ligand mRNA and consequent soluble FasL protein secretion. This, in turn, triggers $CD8^+$ cells apoptosis by FasL/Fas interaction. In this paper we show that the binding of sHLA-I to CD8 membrane molecules induces release of TGF-b1. Pretreatment of cells with anti-CD8 monoclonal antibody inhibits this phenomenon. TGF-b1 secreted molecules are mainly ex-novo synthesized as suggested by the increase in mRNA coding for TGF-b1, or by actinomycin-D pretreatment inhibitory effect. Finally, TGF-b1 molecules are released mainly in bioactive form, as shown by the absence of latent TGFβ-binding proteins in the immunoenzymatic determination without acid-treatment-step and by the immunomodulatory effects of supernatant of our experimental model on cells with specific TGFβ surface receptors. Collectively, these data, further suggest that sHLA-I molecules may down-regulate immune responses by inducing apoptosis in activated $CD8^+$ cells and by inducing TGF-b1 release by apoptotic cells, contributing to immunosuppressive milieu and to resolution of inflammatory and immune response.

INTRACYTOPLASMATIC KIR DETECTION

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NK cells express different HLA class I (HLA-I) inhibitory receptors. In particular killer immunoglobulin-like receptors (KIRs) are crucial both in preventing NK cytotoxicity against self and in "licensing" NK cells to kill HLA-I negative cells. These receptors inhibit NK cells function by binding HLA-I in (inhibition in trans configuration) on target cells. However, the simultaneous expression of both HLA-I and HLA-I inhibitory receptors in each NK cell suggests the possibility of a receptor-ligand interaction within the same cell (cis-association). Cis-interactions with MHC-I have been shown to reduce the surface expression of Ly49 molecules in mice and might also be relevant for some human KIRs. By analogy with the T cell developmental mechanism, NK licensing process is believed to be driven by still unknown educating cells that would present the KIR ligand to the differentiating NK cells. However, an intracellular cis-association between KIRs and MHC-I, able to influence NK cell licensing, is suggested by the evidence that KIR3DL1*004, a KIR not expressed on the cell surface, is a protective allele against HIV progression in individuals expressing its ligand (Bw4 alleles). In order to investigate the eventuality of an intracellular cis KIR-HLA-I interaction, we have tested different anti-KIR mAbs (CD158a and b). After the labelling of surface KIRs with unconjugated mAbs, fixation/permeabilization and intracellular staining were performed using PE-conjugated mAbs. Results suggest the intracytoplasmatic presence of both CD158a and b regardless the presence of cognate KIR-ligand.

$CD4^+CD26^-CD38^+$ T-CELLS: ROLE OF NEW DISCRIMINATING MARKER IN THE IMMUNODIAGNOSIS OF HODGKIN LYMPHOMA

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Introduction : Diagnosis of Hodgkin Lymphoma (HL) by flow cytometry (FC), in lymph node (LN), has been largely unsuccessful because of its failure to identify the neoplastic within a mixed infiammatory background. Therefore, most FC studies have examined the lymphocytes (predominantly $CD4^+$ T) surrounding neoplastic cells and tested the activation markers for try to identify specific features that distinguish HL infiltrates from Reactive Lymphoid Hyperplasia (RLH). Our study investigates and compares the FC immunophenotype and the activation markers, as CD26 and CD38, on $CD4^+$ T from LN cell suspension from HL and RLH.

Methods: Cell suspensions of fresh mechanically disaggregated LN (58 involved by HL and 52 by RLH) were stained with a set of monoclonal antibodies to lymphoid antigens and analyzed by FC to assess the expression of T-cell antigens.

Results: Statistically significant differences were observed for activation markers between HL and RLH for $CD38^+$ (43% vs 18%) and $CD26^+$ (15% vs 50%) on $CD4^+$ T cells. So the $CD4^+CD26^-CD38^-$ T cell is a more prevalent population in HL than RLH.

Conclusions: Since a $CD4^+CD26^-CD38^+$ profile appears restricted to reactive infiltrate in all HL subtypes the FC could be a useful and practical adjunctive tool in the diagnosis of Hodgkin Lymphoma.

PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF HUMAN MEMORY TH17 CELLS IN SYSTEMIC SCLEROSIS

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Systemic sclerosis (SSc) is a connective tissue desorder characterized by fibrosis and vascular changes in the skin and internal visceral organs, with an autoimmune background. Transforming growth factor-β (TGF- β) is considered to play a central role in the pathogenesis of SSc. Recently several reports showed TGF- β and Il-6 induce development of the Th17 lineage, a subset have been shown to play a crucial role in the induction of autoimmune tissue injury. Since TGF- β and Il-6 have been considered as crucial cytokines in SSc, Th17 response could be involved in the pathogenesis of SSc. To this issue we analyse this subset within the memory T cell pool both in pherypheral distrect and in fungal specific repertoire from normal controls and SSc patients. By multicolour flow cytometry we correlate the cytokine profile with pattern of receptor expression that have been associated with Th17 cells (CCR6 and CD161). In particular CCR6 has been associated with the trafficking of T, B, dendritic cells to epithelial cells and recognize a chemokine ligand, CCL20, selectively expressed from skin and mucosas.

The results showed: a) a statistically increase of ex-vivo frequency of IL-17 -producing cells in TCD4+CCR6+ subset from SSc patients; b) an enrichment of Th17 cells in fungal specific TCD4+ lines associated with CCR6 expression in SSc patients. Our data reveal a connection between the Th17 response and chemokine system, that can be relevant for understanding the mechanism of IL-17- induced inflammation.

DIAGNOSTIC UTILITY OF CD38 EXPRESSION ON CD8 T CELLS TO EVALUATE ANTIRETROVIRAL THERAPY RESPONSE IN HIV-1 INFECTED YOUTHS

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Surrogate markers for monitoring immuno-virological discordant responders, in addition to plasma viral load and CD4 cells, are still lacking. We assessed the diagnostic utility of CD38 expression on CD8 T cell assay alone or in association with lymphocyte proliferation to mycotic antigens, in evaluating antiretroviral response.

Twenty-eight vertically HIV-infected youths, twenty-one HAART and seven 2 nucleotide reverse transcriptase inhibitors treated, were enrolled in a retrospective study. Responders (57.1%) and Non-responders (42.9%) to stable antiretroviral therapy for a minimum of six months, on the basis of viral load and CD4 T cells, comprehensively evaluated by CD38 expression on CD8 T lymphocytes (CD38 antibody bound per CD8 T cell (CD38 ABC) and %CD38+ of total CD8 T cells (CD38/CD8)) and lymphocyte proliferation to *P. jiroveci*, *C. albicans*, *C. neoformans*, *A. fumigatus* at a single time point after treatment, were selected.

CD38 expression $\geq$2401 CD38 ABC and $\geq$85% CD38/CD8 cutoff points, accurately discriminates Responders versus Non-responders, both measures resulting in 75.0% (CI 42.8-94.5) sensitivity (identification of Non-responder) and 93.8% (CI 69.8-99.8) specificity (identification of Responder), when considered as single assays. The association "$\geq$2401 CD38 ABC or $\geq$85% CD38/CD8" improved sensitivity to 83.3% (CI 51.6-97.9), while the association "<2401 CD38ABC (or <85% CD38/CD8) and lymphoproliferative response positive $\geq$ 2 tested organisms " improved specificity to 100% (CI 79.4-100).

CD38 expression and mycotic antigen-specific T cell proliferation may be used as additional parameters to existing criteria to evaluate antiretroviral response in immuno-virological discordant patients.

ALZHEIMER DISEASE AND LEWY BODY DEMENTIA CAN BE DISTINGUISHED BY THE CYTOMETRIC IDENTIFICATION OF A NOVEL DIAGNOSTIC BIOMARKER

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Alzheimer's Disease (AD) and Dementia with Lewy bodies (DLB) are the most common neurodegenerative dementia in the aged population. Several studies demonstrated that DLB diagnosis accuracy is not satisfactory because its some 'core' clinical features overlap with AD. DLB tends to be under-diagnosed and misdiagnosed as AD. It is important to differentiate the two diseases because DLB patients are more sensitive to adverse effects of neuroleptics, exhibit faster progression and different response to acetylcholinesterase inhibitors. The pathogenesis underlying AD remains unclear and it is controversial whether AD results from a primary abnormality in amyloid precursor protein (APP) or deregulation of the inflammatory system. Several lines of evidence implicate abnormal processing of APP to generate excessive Amyloid-β (Aβ), which precipitates into the extra-cellular space in the brain, forming Aβ plaques. DLB is a disorder characterized by the presence of inclusion bodies, or Lewy bodies (LBs), filled with aggregates of α-synuclein. Some previous reports suggest the presence of peripheral Aβ-specific T cells in AD patients. In this study, for the first time, we analysed the features of peripheral Aβ1-42-specific T cell subsets in AD; we also checked the reactivity to Aβ1-42 peptide in peripheral T cells from DLB patients. We found the presence of Aβ1-42-specific T cells, characterised by a bright level of Protein Kinase C (PKC) phosphorylation as well as a high level of cytokine production, only in AD patients. We believe that these findings may be of help in possible attempts to develop further diagnostic strategies useful for the characterization of AD.

GHSR AND AGING

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The ageing is characterized either by an irreversible loss of the full effectiveness of metabolic process or by a significant hormonal change such as a decrease of GH and IGF-1. This could be due to decrease of GH secreting cells in the pituitary gland or to modification of the GH secretagogue peptide ghrelin (GHS). Ghrelin is a 28-amino-acid peptide. Ghrelin circulates as both desacyl and acylated forms. This n-octanoyl acylation on one of its serine residues (Ser3) is unique to ghrelin and is necessary for the binding of ghrelin to the growth hormone secretagogue receptor (GHSR). The ghrelin receptors were traditionally thought be highly expressed in the pituitary and the CNS and organs systems including immune cells. The gene encoding the GHSR has two splice variants: the full-length GHSR-1a and its truncated molecule GHSR-1b, which contains only five transmembrane domains. GHSR-1a is the receptor to which ghrelin binds and through which ghrelin exerts its effects on growth hormone release; the physiological function of GHSR-1b remains to be characterized. The purpose of our work was, using the monoclonal antibody produced by us and characterized, to study the involvement of ghrelin in the aging process through evaluation of the modulation of its receptor in animal cells and in human peripheral lymphocytes. In our laboratory we produced two sets of monoclonal antibodies specific for GHSR1a and for both isoforms to study the expression of the GHSR in rat splenocytes, cells from lymph nodes and human peripheral blood (PBL) from donors of different age. In both models we demonstrated, with citofluorimetry analysis, a significant decrease of

GHSR in elderly subjects while young and middle-age subjects did not show any significant differences to suggest that the decrease of ghrelin stimulation is a sudden rather then a gradual phenomenon beyond which ageing takes place.

POLY (ADP-RIBOSE) POLYMERASE-1 INACTIVATION PROMOTES REGULATORY T CELL DIFFERENTIATION

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$CD4^+CD25^+$ regulatory T cells (Treg) contribute to the maintenance of immunologic self-tolerance by inhibiting the activation of auto-antigen reactive T cells. Treg cell development occurs in the thymus, is dependent on Foxp3 expression and believed to be promoted by high affinity TCR-MHCII peptide complex interactions. Growing evidence is unveiling a role for poly (ADP-ribose) polymerase-1 (PARP-1) in the regulation of inflammatory/immune responses. In the present work we extended our studies on the effects of PARP-1 inactivation in Treg cell differentiation. Increased numbers of regulatory $CD4^+CD25^+$/$Foxp3^+$ T cells were found in thymus, spleen and lymph nodes of PARP-1KO mice as compared to WT controls. The increased Treg cell frequency at periphery resulted in impaired CD4 cell proliferation and IL-2 production, which could be restored by $CD25^+$ cell-depletion. Treg cells from KO and WT mice displayed no differences in phenotype (CTLA4 and GITR expression) and function (suppression of cytokine production and cell proliferation), indicating that PARP-1 affects Treg cell differentiation rather than function. Purified naïve CD4 cells from PARP-1KO mice stimulated *in vitro* indeed expressed Foxp3 mRNA at higher level and generated a higher number of $Foxp3^+$ cells (inducible Treg cells) than the WT counterpart. PARP-1 KO and WT induced Treg cells displayed similar features (phenotype and anergic state), suggesting that modulation of PARP-1 might be used to induce higher numbers of functional Treg cells. Our findings represent the first evidence that PARP-1 affects Treg cell differentiation potentially opening new perspectives in the modulation of immune responses.

MEASUREMENT OF THYMIC OUTPUT BY MULTICOLOR FLOW CYTOMETRY

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Recent advances in multicolor flow cytometry have enabled a more comprehensive characterization of human thymic output, provided powerful tools to assess naïve and memory T cell pools and therefore the mechanisms of T cell reconstitution. Expression of CD45RA and CD62L has been most useful in humans to measure of naïve T cells. McFarland et al suggested the additional use of CD103 as marker of naïve CD8+ T cells.

We investigated the activity of the immune system in 2 children with DiGeorge Sindrome (FT and PM, aged 7 and 10 years, respectively) and in one thymectomized child over 6 months of age (DI, aged 3 year), by measuring the phenotype of lymphocytes and the response of T cells following in vitro phytohemoagglutinin (PHA) stimulation. Six-color flow cytometric analysis is performed using a BD FACSCanto II flow cytometer (Becton Dickinson).

As compared with control healthy children, all patients have normal TCRαβ expression and proliferative responses. PM and DI display a reduction of both CD4+ and CD8+ T cells and reduction of CD45RA+CD62L+ naïve cells in both CD4+ and CD8+ T cell populations.

FT has normal T-cell numbers suggesting that there may be compensatory mechanisms serving to sustain T-cell counts. He show a substantially high proportion of CD4+ naïve T cells (88.3%) whereas the naïve CD8+ population is absent (0.3%); in the CD8+ population a high proportion of cells display the central memory CD45RA-CD62L+ phenotype (60.6%). However, the relatively high proportion of cells with the phenotype CD8+CD62L+CD103+ in this patient seems puzzling. It has been speculated that these cells come from the gut epithelium, CD103 being an epithelial retention receptor.

Our preliminary data suggest that peripheral T homeostasis is maintained at minimal levels mainly by extrathymic expansion of existing naïve T cells in the periphery or by extrathymic production.

IMMUNOLOGICAL EFFECTS OF THE ANTI-VEGF THERAPY INFLUENCE THE PROGRESSION FREE SURVIVAL OF ADVANCED COLORECTAL CANCER PATIENTS

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Background – A strict correlation was discovered in vivo between the VEGF levels and the impairment of dendritic cells (DCs) in metastatic colorectal cancer (mCRC) pts. Bevacizumab (BEV) addiction to chemotherapy (CT) may improve the number and function of blood DCs. We have focused on the correlation between this immunological favourable effect and the clinical efficacy of a multicyclic BEV-based, 1st-line treatment for mCRC.

Material and methods – Starting from January 2007 we performed a flow cytometric analysis of PB lymphocytes and DC subsets (DC1 and DC2) in 53 mCRC pts who had not received prior CT for metastatic disease or for whom 6 months had relapsed since adjuvant CT (M/F: 31/22, median age: 59yrs; range 32-75; ECOG PS <2), before and every 3 courses of a BEV+CT (5-FU± CPT11± Oxaliplatin) program. Biological data of the 42 evaluable pts that received all the planned treatment were correlated to both tumor response (OR) and progression free survival (PFS).

Results - During treatment, DCs and their subsets showed a progressive, significant increase in absolute number, with respect to baseline, both in responder (CR,PR,SD) (67%) and in non responder pts; only responder pts keep this immunological effect at the moment of clinico-radiological revaluation, performed at 3 weeks since the last course administration. The DC and DC1 absolute number of pts with PFS $>$ 15 months (58%) increased more evidently during antiangiogenetic-therapy and was significantly higher after therapy completion with respect to DC of pts with shorter PFS ($p < .02$).

Conclusions - First-line BEV-based therapy in mCRC pts improves the number of blood DCs, pointing out a potential additional anticancer mechanism of this drug. Because, from our data, the recovery of DC correlates with longer PFS, we could hypothesize that BEV influence tumor regrowth by contributing to overcome the impairment of the host immune surveillance induced by VEGF.

IMMUNOLOGICAL EFFECTS OF POLYPHENOLS CONTAINED IN FERMENTED GRAPE MARC (FGM) ON HUMAN HEALTHY PERIPHERAL BLOOD CELLS

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Polyphenols from red wine when ingested in regular and moderate doses (e.g. two glasses of wine per day) are able to exert beneficial effects in humans.

In particular, according to current literature and our recent studies, vasodilatation due to the release of nitric oxide and modulation of the immune responsiveness seem to contribute to the cardio-protective, anti-aterogenic, anti-inflammatory and anti-neoplastic effects played by red wine polyphenols.

Here, we have evaluated the in vitro effects of fermented grape marc (FGM) from two cultivar, Negroamaro (Italy) and Koshu (Japan), on normal human peripheral blood mononuclear cells.

When used either solubilized in water or in ethanol both FGMs could induce intracellular expression of cytokines [interleukin (IL)-4, IL-8, IL-10, IL-12 and TNF-α], whose extent varied according to the different composition in flavonoids from the two preparations.

Flavonoids contained in Koshu and Negroamaro induce a detectable expression of intracellular IL-12 in human monocytes and, in some instances, values are higher than those observed in LPS-treated samples.

Koshu and Negroamaro induce expression of IL-10 in monocytes while they inhibit its generation in the presence of LPS. The same is true in the case of IL-10 production from lymphocytes activated with both Koshu and Negroamaro FGMs. This effect may be explained by postulating the presence of receptors on lymphocyte membrane for both flavonoids and PMA whose stimulation gives rise to a cooperative action rather than to a competion.

With special reference to pro-inflammatory cytokines, IL-8 expression is enhanced by both FGMs as well as inhibited in the presence of LPS.

In relation to intracellular TNF-α experiments, Koshu FGM exhibits a clear-cut pattern of response in the sense that it promotes generation of this cytokine and inhibit its production in the presence of LPS. Also Negroamaro FGM induces expression of TNF-α but lacks the inhibitory capacity when co-cultured with LPS.

IL-4 presence in lymphocytes is concerned, Negroamaro in all forms and Koshu in water are unable to generate this cytokine, while Koshu in ethanol can perform this activity. This experiments suggests that the mixture of flavonoids contained in a given preparation can regulate, even if to a different extent, production of a selective cytokine.

Our data clearly show the ability of both Koshu and Negroamaro solutions to modulate the in vitro immune response.

Key words: cytokine, Fermented Grape Marc, immunity, interleukin, wine

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NK CELLS IN PREGNANCY

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Decidual NK (dNK) cells represent the predominant lymphocytes in the uterus during early pregnancy. dNK cells are phenotypically and functionally distinct from their peripheral blood counterpart (pbNK) as they are CD16 negative and poorly cytotoxic while they constitutively secrete a number of cytokines, chemokines and angiogenic molecules. Particular attention has been recently devoted to understand the importance of NK cells in the control of pregnancy outcome. The close encirclement of spiral arteries by NK cells together with their ability to produce angiogenic factors suggest that they might influence mucosal vascularization. On the other hand, their proximity to the extravillous trophoblast supports the idea that dNK cells could regulate its invasion during placentation. The origin of dNK cells is presently unknown. They may arise from NK cell progenitors present in the uterus or recruited from other tissues, and/or from NK cell populations recruited from blood.

In this regard we have evidence showing that chemokines present in the uterus can support pbNK cell migration throughout endothelial and stromal decidual tissues as well as dNK cell migration through stromal cells and that pregnancy associated factors acting at systemical and local level including hormones and/or inflammatory cytokines can tightly control this process. This observation correlated with the ability of progesterone to positively modulate stromal cell chemokine expression. Notably, when pbNK cells are cocultured with decidual stromal cells they acquire a chemokine receptor profile resembling that of dNK cells.

Thus it can be suggested that pbNK cell recruitment to the uterine compartment can contribute to the

accumulation of dNK cells and once in the uterus, pbNK cells acquire a specific phenotypic and functional profile to ensure a good outcome of pregnancy.

IMMUNOSUPPRESSIVE EFFECTS OF MESENCHYMAL STEM CELLS FROM HUMAN AMNIOTIC FLIUD: INVOLVEMENT OF CD4+CD25+ REGULATOTY CELLS.

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Objective: Mesenchymal stem cells (MSCs) have been shown to be able to escape immune recognition and inhibit immune responses. One of the mechanisms for the inhibition of immune-cells function seems to be the production of soluble factors by tumour cells that enhance the frequency of T regulatory cells (Treg). CD4+CD25+ regulatory cells have strong immunomodulatory potential. The objective of this study was to investigate the effect of MSCs from human amniotic fluid (AF) and bone marrow (BM) on the development of regulatory T cells.

Methods: Peripheral blood mononuclear cells (PBMCs) from healty donors were exposed to MSCs isolated from human AF and BM in 10:1 ratio for 5 days. The induction of T reg cells in PBMCs was determined by measuring the proportion of CD4+CD25+ T cells in all CD4+ T cells by flow cytometry.

Results. The percentage of CD4+CD25+ T cells was analyzed by flow cytometry when PBMCs were co-cultured with MSCs in the presence of IL-2 (300U/ml). The control group was represented by PBMCs cultured in presence of IL-2 (300U/ml). We found that the CD4+CD25+ regulatory T cells significantly increased in the presence of MSCs. The percentage of CD4+CD25+ T cells was 5.5% in the control group, 23.4% in PBMCs co-cultured with AF-derived MSCs and 16% in PBMCs co-cultured with BM-derived MSCs.

Conclusion. Our data suggest that human AF represents a rich source of MSCs. These cells shown a stronger immunosuppressive effect compared to the BM-derived MSCs. This effect was mediated by inducing the generation of CD4+CD25+ regulatory cells.

CYTO-CHEMOKINES AND TREG IN RCC PATIENTS

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RCC (Renal Cell Carcinoma) accounts for 3% of male cancer and 2% of female cancer. 20-30% patients present with metastatic disease and 20-40% patients develop metastatic disease following nephrectomy for localized disease. In RCC patients there is a well documented shift from a type-1– to a type-2 cytokine response. In fact patients rendered disease-free by primary tumor excision and/or immunotherapy revert to a predominance of IFN- g producing type-1 CD4+ T cells. Sunitnib was showed to reverse of Type-1 immune suppression and decreases Treg cells in RCC patients and to reverse myeloid cell-mediated immunosoppression in patients with mRCC. Aim of the work is evaluation of T regulatory cells (Tregs) and serum cytokines in 90 patients affected by RCC and 20 patients with mRCC in treatment with Sunitinib. Tregs were analized by flow cytometry using antibodies against CD3, CD4, CD8, CD16, CD25, CD56, CD152, CD184, CD279 and FOX-p3. The simultaneous quantitative analysis of 27 and 23 differents cytokines is determinated by Bio-Plex suspension array system. Preliminary results demonstrate that statistically significant different cytokines levels were detected. Also Tregs subsets were statistically significant different. Preliminary data support the cytokines screening are suitable to identify immunologic profiles and biomarkers predictive of prognosis and clinical response. Ongoing studies are evaluating correlations between cytokines and Treg profiles with the patients outcome and response to treatment in mRCC patients.

SUGGESTIONS FOR DIAGNOSING HYPER-REACTIVITY TO LOCAL ANESTHETICS BASED ON A FLOW CYTOMETRIC STUDY

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Identification of the antigens responsible for allergic reactions is essential both for diagnostic purposes and for an effective prevention. Allergic reactions towards local anesthetics may also be caused by the stabilizing agents used in preparations with adrenaline as vasoconstrictor. Aim of our work was to demonstrate that a complete diagnosis of hypersensitivity to such drugs should also include the analysis of the minor vial ingredients. Subjects referring allergic reactions to local anaesthetics were enrolled and their blood samples assayed for both the active principle and the additives (K-metabisulphite).

Basophil Activation Tests used for revealing subjects' reactivity have been validated for *in vitro* diagnostics (CE-IVD). They are based on the flow cytometry detection of CD63 and CD203c markers following cells incubation with the selected allergen. Degranulation triggers CD63 expression on cellular surface, release of preformed mediators (histamine etc.) as well, while CD203c becomes over-expressed in response to allergens.

The study was performed within IRMA laboratories on 63 patients referring a previous anaphylaxis history towards local anaesthetics with adrenaline. From the group testing lidocaine, 14 subjects on 32 were sensitive to the E224, 9 of which did not react to the active principle itself as expected. 22 individuals tested mepivacaine: 12 were positive to E224, 6 of them did not show reactivity towards mepivacaine. On 9 testing articaine, 2 patients were positive to E224 only.

BATs revealed a good level of reliability in the detection of drug hyper-reactivity (62.5% for lidocaine sensitivity, 72.27% for mepivacaine, 55.5% for articaine), being thus suggested for the baseline diagnosis of suspected sensitization. Our results also pointed out the need for widening the analysis towards additives of food and drugs.

DEFINING THE ROLE OF INNATE IMMUNITY IN MULTIPLE SCLEROSIS: HINTS FROM POLYCHROMATIC FLOW CYTOMETRY

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Dendritic cells of the innate immune system and other professional antigen-presenting cells like B-cells and monocytes/macrophages sense exogenous pathogen associated molecular patterns and endogenous danger signals to initiate, sustain and regulate immune responses against microbes and tumor and necrotic cells. This ability mainly relies on a family of conserved membrane-associated receptors, the Toll-like receptors. In recent years a great body of evidences have demonstrated a role for the innate immune compartment and pathways in the onset of autoimmune diseases through cytokine production, antigen presentation and costimulation. In order to appreciate possible mechanisms for innate triggering of autoimmunity, we are performing multiparametric flow cytometry to study intracellular accumulation of cytokines in plasmacytoid (HLA DR+ Lin - CD123+) and myeloid (HLA DR+ Lin - CD11c+) dendritic cells, B-cells (CD19+) and monocytes (CD14+) upon toll-like receptor stimulation with a panel of natural and synthetic compounds in whole blood from healthy subjects and from Multiple Sclerosis. This technology allows to measure cytokine production from distinct cellular subsets simultaneously, reproducibly, and swiftly, reducing sample manipulation to the minimum, and providing data with high information content. The cytokine profiles of these cellular subsets and the discrepancies between healthy subjects and MS patients will be discussed.

POLYCHROMATIC FLOW CYTOMETRIC ANALYSIS OF T CELL SUBSETS DEFINE USEFULL BIOMARKERS TO CLINICAL ACTIVITY EVALUATION OF MULTIPLE SCLEROSIS PATIENTS

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Multiple sclerosis is an inflammatory demyelinating disease of the CNS, although the involvement of the immune system is widely accepted the underlying pathogenetic mechanisms remain poorly defined. We and others have shown that in addition to the CD4+ alpha/beta effector autoreactive T cells, CD8+ alpha/beta T cells and gamma/delta T cells also are involved in disease pathogenesis. Moreover recent research in the field of immune regulation has focussed on a population of T cells which are able to actively suppress immune responses, and which are likely to play a major role in the control of the activation of autoreactive lymphocytes and in the induction and maintenance of peripheral tolerance. These cells, appropriately called regulatory T cells, have been shown to be functionally deficient in patients with MS. In this study we have established a sophisticated analysis by polychromatic flow cytometry (8 colours) in order to study simultaneously all ex vivo isolated effector and T reg cell subsets from MS patients and healthy controls. In the attempt to define phenotypic and functional correlations with the clinical state of MS patients we monitored several different biomarkers on effector alpha/beta, gamma/delta T cells and on Treg cells in patients in different phases of the disease and in healthy donors. Patients in the stable phase of the relapsing-remitting form of the disease had reduced numbers of CD39+ T reg cells within the $CD4+CD25^{high}$ cell population whereas the distribution and frequency of gamma/delta effector CD16+ T cells in MS patients was significantly different in the acute phase of disease compared to the stable phase and to that of healthy individuals.

GAMMADELTA T CELLS WITH A TH1/TH17 PHENOTYPE ARE EXPANDED IN HIV-! INFECTED PATIENTS AND RESPOND TO CANDIDA ALBICANS

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Two main subsets of gammadelta T cells are known: Vdelta2 T lymphocytes, circulating in the peripheral blood, are involved in the response to mycobacteria and certain viruses, while Vdelta1 T cells are resident in the mucosal-associated lymphoid tissue and participate in the immunity against intracellular microrganisms. Vdelta2 T cells recognize non-peptidic phosphorylated metabolites of isoprenoid biosynthesis expressed by mycobacteria, whereas Vdelta1 T cells mainly interact with MHC-related antigens (MIC-A, MIC-B) and with receptors, called UL-16 binding proteins, for the UL-16 protein produced by cytomegalovirus-infected cells. Vdelta1 T cell clones can release IFN-gamma upon challenge with $MIC\text{-}A^{+}$ cells, while it is produced by the Vdelta2 T cell subset upon stimulation with non-peptide antigens.

We show that: i) a population of circulating Vdelta1 T lymphocyte producing both IFN-gamma and IL-17 is expanded in HIV-1 infected patients; ii) this population is capable of proliferating and enhancing cytokine production in response to Candida albicans, while Vdelta2 T cells respond to mycobacterial antigens; iii) IFN-gamma/IL-17 double producers express the RORC and the TXB21 transcription factors, the CCR7 homing receptor, the CD161 molecule involved in transendothelial migration, and the CCR4 and CCR6 chemokine receptors. This gammadelta T cell subset

not only produce Th1/Th17 cytokines, but express a number of homing and chemokine receptors, thus being equipped for recirculation through lymph nodes and peripheral tissues.

PRODUCTION OF SOLUBLE HLA-G MOLECULES BY MESENCHYMAL STROMAL CELLS AFTER IN VITRO IL-10 ACTIVATION: A MARKER FOR ''A PRIORI'' EVALUATION OF THEIR IMMUNOREGULATORY ACTIVITY

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Graft versus host disease (GvHD) is the main unfavorable evolution of allogeneic hematopoietic cell transplantations (HSCT). Even thought GvHD is now controlled by pharmacologic treatment, recent studies have proposed a beneficial effect of mesenchymal stromal cell co-transplantation (MSCs). These cells are able to inhibit the innate and adaptative cell-mediated immune response with a variable efficacy between MSCs from different subjects. For this the availability of markers of MSC inhibitory activity would be of extreme interest in HSCT. Several soluble factors have been recognized as responsible of MSC immuno-regulation. In our study we have evaluated if HLA-G molecules could be implicated in MSCs functions. HLA-G are non-classical HLA class I molecules implicated in the immune response, inhibiting T CD8+, T CD4+, natural killer, B and dendritic cell activation.

By flow cytometric analysis and immunosorbent assay, we have analyzed the production of membrane-bound and soluble HLA-G by MSCs after IL-10 treatment.

The bone marrow derived (BM) MSCs with or without rIL-10- treatment have been analyzed in particular for IL-10R1 expression by flow cytometry with anti-IL-R1 MoAb. The rIL-10 treatment has increased IL-10R1 expression ranging from 12.0 and 58.3% with a mean value of 32.9 %. To confirm that rIL-10 treatment is involved in IL-10R1 up-modulation the MSC cultures have been pre-treated with an anti-IL-10R1 MoAb. By flow cytometric analysis we observed that this pre-treatment has significantly reduced the up-regulation of IL-10R1, membrane HLA-G1 expression and sHLA-G secretion (ranging from 0.0 and 3.1 ng/ml).

In conclusion, our data demonstrate the role of sHLA-G molecules in the immuno-regulatory effect of MSCs. The *in vitro* treatment with IL-10 induces different levels of HLA-G secretion by MSCs which is seems to be a marker of MSC functionality.

EARLY CD4+ LYMPHOCYTE RECOVERY CORRELATES TO CLINICAL OUTCOME AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Recent reports suggested that early CD4+cell recovery after allogeneic stem cell transplant (SCT) has an strong impact on acute graft versus host disease (aGVHD), overall survival (OS), transplant-related mortality (TRM). We evaluated CD4+ cell count at 20 days after SCT (r. 12-34) on 99 patients (pts), with a median age of 46 years (r.11-67), underwent to bone marrow (23 pts) and peripheral blood (76 pts) SCT. The median follow-up was 46 months (r.12-86). Donors were 83 matched sibling and 16 alternative. Conditioning regimens were myeloablative (48 pts) or at reduced intensity (51 pts). The incidence of aGVHD (grade II-IV) was 44%. Univariate analysis showed that early CD4+ cell recovery is correlated with OS and TRM but not with aGVHD. Roc curve of CD4+ cell count indicated that the cut-off was 115/μl. At 2 years follow-up, pts achieving this cut-off showed significantly lower cumulative TRM respect on pts who did not. At 5 years, OS was better in pts with more than 115 CD4+/μl, respect on pts with less. We evaluated, with multivariate analysis, the predictive role of other factors associated to OS as donor type and sex, AB0 identity, recipient sex and age, stem cell source, conditioning regimen, disease type and status and we found that the main predictive factor for clinical outcome after allogeneic SCT is represented by early T helper count. Patients with low early CD4+ count need to be followed more carefully to avoid transplant complications. The graft manipulation may represent an opportunity to obtain an improvement in early immune recovery and overall survival.

METHODOLOGY AND TECHNOLOGY

MICROSCOPIC EVALUATION OF PHAGOCYTIC ACTIVITY OF HUMAN MACROPHAGES AGAINST ASPERGILLUS CONIDIA AFTER IMMUNO-STIMULATING TREATMENT

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Aspergillus species are recognized as major fungal pathogens in severely immuno-suppressed or neutropenic patients, in which invasive pulmonary aspergillosis (IPA), characterized by hyphal invasion and destruction of pulmo-

nary tissue, is the most common manifestation of an *Aspergillus* infection. Airborne transmission of fungal spores is the major route of *Aspergillus* infections and resident alveolar macrophages constitute the primary immune defence for detection and elimination of *Aspergillus* conidia. Here we evaluated, using microscopic techniques, the effect of the immuno-stimulating agent Thymosin α1 (Tα1), a naturally occurring thymic peptide used worldwide for the treatment of some immunodeficiencies, malignancies, and infections, on the phagocytic ability of human monocyte derived macrophages (MDMs) against conidia of *Aspergillus niger*. By confocal microscopy, we analysed the influence of Tα1 on adhesion and internalization of *A. niger* conidia stained with Alexa Fluor 488 succinimidyl ester, a fluorescent probe that selectively link to primary amines located on live cell surface proteins. We also performed an ultrastructural analysis of conidia adhesion and internalization by scanning (SEM) and transmission (TEM) electron microscopy, respectively. Results indicated that Tα1 induced morphological activation of MDMs, also dramatically stimulating their phagocytic response. Actually, Tα1 is able to increase, already after 30min of treatment, the number of conidia adherent to cell membrane or internalized by MDMs, compared to the untreated control, as demonstrated by SEM and confocal microscopic observations. TEM analysis confirmed the presence, in treated MDMs, of an augmented number of internalized conidia in both resting and swollen stages.

ISOLATION AND CHARACTERIZATION OF CULTURED PLACENTA-DERIVED STEM CELLS

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Background: Regenerative medicine needs a safe and ethically acceptable stem cell source for the development of new therapeutic strategies. Human term placenta may represent an attractive candidate.

Aims: to optimize the isolation of human amniotic epithelial cells (hAEC) from term placenta obtained from caesarean section procedures; to characterize and maintain hAEC in long term cultures.

Methods: The amnion was stripped from the underlying chorion and digested with trypsin. The isolated hAEC were seeded on collagen coated flasks (1×10^5cells/cm^2) in DMEM with 10% FBS and 20 ng/ml EGF. Morphology, flow cytometry and immunofluorescence analyses were evaluated on cultured cells at the 1st, 3rd and 5th passage.

Results: Four amniotic membranes were dissociated immediately after delivery. At least 1.5×10^6 cells were recovered in each isolation with a viability of 75-80%. At first split, cultured cells displayed a rounded cobblestone appearance with a high coexpression of epithelial stem cell marker EpCAM (78-94%) and CD49f (91-98%). This feature was maintained until the third passage, when cells underwent epithelial to mesenchymal transition acquiring a more spindle-shaped morphology and expressing typical mesenchymal markers, such as CD90 (33-98%), CD105 (38-67%), and S100A4 (80-85%). However, the expression of epithelial markers CD73 (95-99%), CD166 (16-98%), CD13 (84-90%), cytokeratin 18 (>95%), cytokeratin 19 (85-90%) and alphafetoprotein (80-90%) were maintained.

Conclusions: Human amnion contains stem cells that maintain epithelial characteristics until third passage in culture. Further studies are needed to optimize hAEC isolation and to test their capability to differentiate into mature epithelial cells suitable for the development of new and more effective strategies for regenerative medicine.

A MODULAR PLATFORM FOR CELL CHARACTERIZATION, HANDLING AND SORTING BY DIELECTROPHORESIS

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The physical manipulation of biological particles is of vital importance in the development of miniaturized lab-on-chip devices. Dielectrophoresis (DEP) is a method for cell handling and sorting without physical contact, exploiting the dielectric properties of cells suspended in a microfluidic sample, under the action of high-gradient electric fields. The dielectrophoretic platform is composed of several functional units, organized in a first characterization module and in a series of manipulation stages that can be rearranged on a single chip, depending on the target application. The non-uniform electric fields are generated by microelectrodes patterned on the silicon substrates of microfluidic channels using micro-electro-mechanical-systems (MEMS) technology. Numerical modelling has been performed to simulate the electric field distribution and to quantify the pico-Newton forces at the microscale. From cell motion analysis in the characterization stage, cell permittivity and conductivity can be determined as functions of frequency, whose knowledge is essential for the design of the electric excitation in order to obtain the desired effect in the handling modules. The manipulation modules achieve several functionalities: the multi-bar array module can be used as a selective cell filter, or as a cell conveyor stage, depending on the phase shift between consecutive electrodes excitation; the focusing stage allows the alignment of a cell population along the axis of the microfluidic channel, where a caging module can trap cells for a subsequent in-situ fluorescence analysis of labelled membrane proteins; the deviation stage can be activated to move only selected cells in a dedicated microfluidic outlet; the spiral array module acts as a selective cell concentrator, allowing the direct observation of filtered cells at the center of the biochip.

INTEGRATION OF TIME-LAPSE LIVE CELL IMAGING AND FLOW CYTOMETRY IN CELL PROLIFERATION STUDIES

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Cell proliferation has been studied for a long time by flow cytometry (FC) or time-lapse live cell imaging (TL). The two platforms, considered singularly, produce data that convey a piece of the information, FC focusing on distributions of cells in G_1, S, G_2M cell cycle phases, TL on lineage trees following cells in subsequent generations. The present study demonstrated the possibility of a full reconstruction *in silico* of the cell cycle progression considering together the data obtained with both platforms. With this method we disclosed the heterogeneity of the response of cancer cells to X ray exposure, demonstrating that some cells were intercepted by G_1,S, G_2M checkpoints before dividing (generation 0), others after one or even two mitoses (generation 1 and 2 respectively). Some cells experienced repeated delays in different phases and generations. The fate of the cells was also heterogeneous, even within the same lineage, some descendant remained definitively arrested (particularly in G_1 in generation 1 and 2), some refused originating polyploid cells and others died.

CONFOCAL MICROSCOPE ANALYSIS OF SAOS-2 CELLS GROWN ONTO A GELATIN-BASED CRYOGEL SURFACE

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The modification of a gelatin-based cryogel surface plays an important role in bone tissue engineering. We have followed a biomimetic strategy where electromagnetically stimulated SAOS-2 osteoblasts proliferated and built extracellular matrix on a gelatin-based cryogel surface. Moreover, increasing evidence suggests that an electromagnetic stimulus can modulate bone histogenesis and calcified matrix production *in vitro* and *in vivo*. Our aim was to investigate the effects of an electromagnetic wave (intensity of magnetic field, 2 mT; frequency, 75 Hz) on human SAOS-2 cells in terms of proliferation and matrix production.

Cells were seeded onto gelatin-based cryogel surfaces, and electromagnetically stimulated ("electromagnetic culture") or not ("control culture"). The gelatin surfaces were washed with phosphate buffer saline, fixed with formaldehyde, and processed for confocal microscope detection of specific bone markers, such as type-I collagen, decorin, and osteopontin.

Confocal microscope analysis revealed that the stimulation improved the cell distribution on the gelatin surface and caused significantly higher fluorescence intensity.

Taken together these data seem to suggest that the electromagnetic stimulation could be used to improve osteoblast growth and calcified matrix development *in vitro*.

CONFORMATIONALLY ALTERED p53: A POTENTIAL PREDICTIVE MARKER FROM MCI TO ALZHEIMER's DISEASE?

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Background: According to the current clinical criteria, definite Alzheimer's disease (AD) can only be diagnosed following neuropathological examination of brain samples, obtained by biopsy or autopsy. Furthermore, when evaluating the intermediate state between normal aging and established AD, known as mild cognitive impairment (MCI), not all MCI patients progress to AD and hence there is a need of a reliable prediction tool able to identify which patients with MCI will progress to AD. The current inability of clinical criteria to accurately identify this at-risk group underscores the importance of developing biomarkers able to potentially supplement the clinical approaches. Recently a role for conformationally altered p53 as a novel candidate biomarker for early onset AD has been described. The aim of our work is to investigate the usefulness of this method especially for younger patients, thus supporting its putative application for subjects with MCI and earlier in the clinical course of AD.

Methods: We used a flow-cytometric approach to investigate the different expression of conformationally altered p53 among MCI, AD and non-AD subjects on peripheral blood cells. Results: We found that peripheral blood cells from MCI specifically expressed increased levels of unfolded p53 compared to age-matched controls. We found that the expression of conformationally altered p53 is age dependent. For our preliminary data analysis we have arbitrarily worked out the related cut-points by linear regression, taking as reference linear fit of controls, thus dividing the subjects in specific age interval segments. Young (<70 years) MCI patients show levels of conformationally altered p53 comparable to those measured in AD patients, but significantly different from subjects of control group.

Conclusions: Our cytofluorimetric approach for conformationally altered p53 protein was able to predict progression to AD in preclinical patients with MCI two years before clinical diagnosis for AD was made. We found that 50% of MCI patients converted to AD after two years from the beginning of recruitment. In this MCI converted group, 65% was predicted based on elevated levels of conformationally altered p53, whereas 14% progressed to AD based on APOE status.

ANALYZING THE ILLUMINATON AND PHOTOBLEACHING DISTRIBUTION TOWARDS MEASUREMENTS OF EFFECTS INDUCED BY SCATTERING

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Non linear optical scanning microscopy has became a useful tool for living tissue imaging. Biological tissues are highly scattering media and this lead to an exponentially attenuation of the excitation intensity as the light travels into the sample. The localization of the maximum 2PE intensity was found to shift closer to the surface far from the focal region and the 2PE imaging depth limit appears strongly limited by near surface fluorescence. In this work we computed the illumination and the photobleaching distribution in order to characterize the effects induced by scattering. The simulations have been performed for different scattering coefficients and different focus depths. Experimental tests have been carried out by imaging, with a medium numerical aperture objective (N.A. = 0.9), thick scattering fluorescent immobile sample (polyelectrolyte gel). Results confirm that in these conditions no photobleaching effects due to scattering occur close to the surface.

A NEW TECHNIQUE FOR TRANSLATIONAL RESEARCH: LASER CAPTURE MICRODISSECTION ASSOCIATED TO REVERSE PHASE PROTEIN ARRAYS

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Laser Capture Microdissection (LCM) associated to Reverse Phase Protein Array (RPPA) is a novel technique to analyze simultaneously postranslational modifications, essential for the cellular homeostasis. LCM incorporates an inverted light microscope and an infrared laser to obtain desired cell from heterogeneous tissue. RPPA is based on immobilized multiple protein lysates printed onto nitrocellulose coated glass slides and probed with a specific antibody; it is useful to compare protein expression across different samples, to know key cell signalling cascades involved in processes driving tumour growth.

We applied these techniques to study the carcinogenetic process in colorectal cancer models. Rat tissues (normal and cancer colon epithelium, lung or liver metastases) have been collected and subjected to LCM; protein lysates obtained from pure cancer or normal cell population and the corresponding total tissue proteins, were printed with RPPA. The phosphorylation state of nearly 80 proteins involved in cell growth, survival, apoptosis and invasion has been analyzed. Our data show that the pathway activation pattern is completely different between microdissected and whole tissue lysates, indicating that LCM should be always used to better understand the signalling driving cancer progression. A characteristic pattern of pathways, totally different between liver and lung, was also recorded, suggesting that the cell cancer state is organ specific and that the microenvironment has a large impact in metastatization. The study of rat and human liver metastases showed a unique clusterization in pathway activation, thus reinforcing the validity of our animal model. The comparative analysis of signalling portraits in hepatic and lung metastasis might reveal pathway changes possible targets of innovative anticancer therapies.

A NEW IMMUNOMAGNETIC SEPARATION APPROACH APPLIED TO THE SEPARATION OF MESENCHYMAL STEM CELLS SUBPOPULATION

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Mesenchimal stem cells (MSCs) have the capability for self-renewal and differentiation into cells with the phenotypes of bone, cartilage, neurons and fat cells (*1*). These features have driven investigators for using MSCs for cell-based therapies to treat several diseases. The most common source of MSCs has been bone marrow, but alternative sources have been explored (*2,3*). Our previous data demonstrate the presence of putative MSCs isolated from ovarian follicular liquid (*4*).

To confirm these preliminary results we have performed new experiments based on a novel immunomagnetic procedure to isolate rare cells in suspension, using Dynal microbeads and a dedicated multiwells magnetic device (5). Cells were isolated from human follicular liquid as a whole samples or nucleated cell fraction separated by density gradient. The experiments were done in parallel on human MSC cells as positive control. For the first experiments we focus on CD44, a specific surface marker on MSCs. Results obtained in few cases (10) allowed to have a purified CD44+ cell subpopulation that can be checked directly by microscope (conventional and fluorescence) at the bottom of the wells. In the whole samples there were less labelled cells as compare to fractioned ones. The possibility to recover the cells onto "coverslips" (posed of the bottom of the wells) is an important advantage for the next steps of immunostaining and/or biological characterization of the recovered cells. Experiments will be soon designed to verify the stemness of these cells, seeding them in culture and inducing differentiation into other cell lineages to assess *in vitro* the plasticity of these putative MSCs.

References:

1. Barry FP, et al (2004) *IJBCB* 36, 568-584.
2. Erices A, et al (2000) Br J Haematol 109, 235-242.
3. Bukovsky A, et al (2005) Reprod Biol Endocrinol 3, 17-29.
4. Riva F, et al (2008) It J Anat Embryol 113 (1,2), 241.
5. Mazzini G, et al (2006) Cytometry 69A (5), 465.

TOPOGRAPHICAL DISTRIBUTION OF PROTEINS ONTO DIFFERENT MODIFIED TITANIUM SURFACES

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Biomaterials and medical devices following implantation acquire a layer of host proteins prior to interacting with host

cells. Thus, it is highly probable that the types, levels and surface conformations of the adsorbed proteins are critical determinants of the tissue reaction to such implants. Conversely, the types, concentrations, and conformations of these surface-adsorbed proteins are dependent on biomaterial surface properties that dictate the adhesion and survival of cells, especially macrophages on protein-coated surfaces. The aim of this study was the investigation of the adsorption of fibrinogen (Fbg), fibronectin (Fn) and human immunoglobulin (HIgG) onto titanium surfaces (TAA and TAAK) modified by different Anodic Spark Deposition (ASD) processes and compared to unmodified titanium surface (Ti). In contrast to Ti sample, SEM observation of TAA and TAAK samples showed their different micrometric surface morphology which is believed to play a role in cell adhesion. Confocal microscopy analysis indicated the topographical distribution of Fn, Fbg and HIgG onto TAA and TAAK and at saturation level, all tested surfaces showed a uniform and homogenous distribution of the tested protein. Some spots, probably corresponding to protein aggregates, appeared on the different materials, but no other visible modification was observed. No changes in morphology of murine macrophage cells (RAW 264.7) attached to TAA or TAAK was shown after 24 hours of incubation. TAA and TAAK can be considered promising modified titanium surfaces for biomedical implants.

SAOS-2 CELLS STIMULATED BY ELECTROMAGNETIC BIOREACTOR ONTO 3D TITANIUM ALLOY SCAFFOLD

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Using an electromagnetic bioreactor (magnetic field intensity, 2 mT; frequency, 75 Hz), we investigated the effects of electromagnetic stimulation on SAOS-2 human osteoblasts seeded onto a 3D titanium (3D Ti) scaffold. After incubation with SAOS-2 cells, SEM images revealed that, because of the electromagnetic stimulation, the cells proliferated over the available surface of the 3D Ti scaffolds; statically cultured cells were few and were essentially organized in a monolayer. The immunolocalization of type-I collagen showed a more intense fluorescence in the electromagnetically cultured scaffold than in the static condition, revealing that stimulation is effective in terms of higher cell proliferation and more intense production of bone extracellular matrix. The immunolocalization of decorin, osteopontin, and osteocalcin confirmed a similar culture structure. In comparison with control conditions, the electromagnetic stimulation caused increased surface coating with type I collagen, osteopontin, osteocalcin, osteonectin. In order to overcome the total immunocompatibility with the patient, human bone marrow-derived MSCs (BM-MSCs) were isolated from adult patient and their osteogenic potential was evaluated onto the same 3D Ti scaffold. The results showed good cell adhesion, proliferation and differentiation in static conditions. Further studies will be performed investigating the effect of an electromagnetic bioreactor onto BM-MSCs adherent to 3D Ti scaffolds.

ONCOLOGY

EFFECT OF EGF ON VEGF EXPRESSION IN COLON CANCER CELL LINE

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Background: Epidermal growth factor (EGF) is a key regulating cell survival and several different studies confirmed this role in the pathogenesis of human cancer. Through its binding to epidermal growth factor receptor (EGFR), EGF activates an extensive network of signal transduction pathways. Moreover, this growth factor might be associated with synthesis and secretion of several different angiogenic growth factors, like vascular endothelial growth factor (VEGF). Infact, in several cancer cell lines EGF as well as abnormal activation of EGFR induce VEGF expression. VEGF plays a major role in tumor angiogenesis, infact it is up-regulated in different types of cancer and its expression is inversely correlated with patient survival in many human cancers, including colon carcinomas. Signal transducer and activator transcription 3 (STAT3) has been identified as a major regulator of VEGF expression in glioblastoma and prostate cancer.

Methods: We investigated whether the treatment with EGF in HT-29 cells could induce an increase of VEGF expression like in glioblastoma and prostate cancer cell line. We measured the effects of EGF on the VEGF mRNA levels by Quantitative Real Time-PCR (qRT-PCR) and in parallel we measured secreted VEGF levels by Enzime-Linked Immunosobent Assay (ELISA). Subsequently, we examined the abundance of nuclear STAT3 in HT-29 treated with EGF by Western Blotting and we conduced Chomatin Immunoprecipitation (ChIP) to assess STAT3 binding to specific motifs in the VEGF promoter. Finally, to confirm STAT3 involvement in EGF-induced VEGF mRNA production, we silenced STAT3 expression using RNA interference (siRNA). Moreover, using LY294002, an inhibitor of the phosphoinositide 3-kinase, we investigated whether PI3K pathway is required for VEGF transcriptional regulation.

Results: We found that EGF up-regulates VEGF expression. Our results suggested, also, that STAT3 binds consensus motifs within VEGF promoters under EGF stimulation in colon cancer cells. All these EGF effects were significantly

blocked when HT-29 cells were treated with LY294002 or with small interfering RNA (siRNA) targeting STAT3.

Conclusions: This study identified the EGF/PI3K/STAT3 signaling as an essential pathway regulating VEGF expression in EGF-responsive colon cancer cells. This suggests that STAT3 pathways might constitute attractive pharmaceutical targets in colon cancer patients where anti-EGF receptor drugs are ineffective.

DETECTION AND DISTRIBUTION OF CANCER STEM CELLS IN SOLID TUMOURS

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Cancer Stem cells (CSCs) hypothesis supports that only a small subset of cells within a tumour is capable of both tumour initiation and sustaining tumour growth. In this preliminary study, we analyzed stemness and differentiation phenotype in 6 types of human cancer such as: breast, head and neck, lung, gastric cancer, melanoma and sarcoma. For breast (40 samples) and head and neck (16 samples) cancer, we used CD44 and CD24 antigens, for lung (133 samples) and gastric (35 samples) cancer, melanoma (16 samples) and sarcoma (20 samples), we used CD133 by flow cytometry as reported in literature. We started from fresh samples obtained from surgery compared to stabilized cell lines. The tissue samples were disaggregated mechanically and immediately tested by flow cytometry, while another part of tissue was digested in a digestive solution (collagenase/dispase) at 37°C for 3-4 hours in order to obtain a cell line. Calu1, A549 and LC31 are stabilized cell lines from Non Small Cell Lung Cancer (NSCLC), Colo 38 is a stabilized cell line from melanoma, MCF-7 is a stabilized cell line from breast cancer, MKN28 and AGS from gastric cancer and MG63 and HT1080 are stabilized cell lines from sarcoma. The results showed, that, after the disaggregation, in breast cancer, the mean percentage of cells $CD44^{+}CD24^{low}$ were 5,8%, in lung cancer the mean percentage of CD133 marker was 6%, in gastric cancer the mean percentage of CD133 was 7%, in melanoma, the mean percentage of CD133 was 2% and in sarcoma was 3% of total cell population. The same results were obtained for stabilized cell lines.

Our data showed that, in the cancers analysed, there was a small cell subpopulation with stemness phenotype, indicating that the tumour can be originated starting from cancer stem cell.

PHENOTYPIC CHARACTERIZATION OF HUMAN PULMONARY BLASTOMA CELL LINE

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Background. Sarcomatoid carcinomas are poorly differentiated carcinomas with a sarcomatoid component and characterized by the epithelium-mesenchymal transition. They include different cancers such as spindle cell carcinomas, giant cell carcinomas and lung pulmonary blastomas. In our study, we have isolated and characterized a human pulmonary blastoma primary cell line termed LC114.

Methods. The tissue has been partially disaggregated and digested in a solution of collagenase/dispase. To obtain a stabilized cell line, three mediums were used: IMDM, BEBM and IMDM/BEBM (2:1). Moreover, to characterize the phenotype of LC114 cell population, CD44, CD29, CD90 and vimentin (mesenchymal markers), CD133 (stem marker), CD326 (EpCAM) and cytokeratins were tested by flow cytometry. Side population and sphere formation were analysed. Cell cycle analyses on both cell line and correspondent paraffin-embedded tissue section of LC114 were performed.

Results. Cytometric analysis showed that CD133 was between 5%-10%, CD90, CD326, CD29 and CD44 markers were 3%, 6%, 85% and 80% of cells, respectively. After 30 days of culture, CD133 levels were increased up to 30%, all cells expressed CD90 and vimentin, CD29 and CD44 remained 80% and CD326 was lost. Cell adhesion was observed only in IMDM/BEBM medium. Initially, the cell population was heterogeneous with epithelial and mesenchymal cells. After 15-30 days, only fibroblast like cells were observed. Probably, the sarcomatoid population was selected in culture. LC114 cells formed spheres and showed a side population. The cell cycle analysis showed an aneuploid population (DI = 1.4) in embedded-paraffin tissue and diploid population in LC114 cell line.

Conclusions. We have selected, characterized and stabilized a primary cell line of pulmonary blastoma and confirmed the presence of a CD133+ stem-cell-like population.

TRANSFER OF MEMBRANE AND CYTOSOLIC LABELLING DURING FASL AND STAUROSPORINE -INDUCED APOPTOSIS

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Apoptosis is an important cell suicide programme involved in physiological and pathological processes and can be induced in different ways depending on cell type and acquired signal. Markers of apoptotic death are caspase activation and PS exposure. In many situations, during their development, activation, and different functions, T and B cells interact with other cellular partners. Although cells are usually considered as entities with relatively stable phenotypes, some physiological processes are now known that may lead to expression of unexpected cell surface. This phenomenon, named trogocytosis, is an active membrane transfer triggered specifically by antigen receptor signalling

and occurring within minutes of conjugate formation between live cells. In this work we have evaluated transfer of membrane and cytosolic labelling during apoptosis in Jurkat T cells treated with FasL or staurosporine. We have evaluated omotypic exchange by means of PKH67, PKH26, DiI, CFSE, Calcein AM and Mitotracker Green FM, taking into account transfer of membrane and cytosolic labelling after 30 and 120 min. Furthermore, we have analysed PS exposure concomitantly to caspase activation, during the same time points. Our results suggest a novel role for Fas and its specific ligand FasL, besides its death-inducing function that is best documented in several cell types. Hence, we show that FasL is able to induce membrane and cytosolic exchange in Jurkat T cells with significant differences to that observed with staurosporine induction.

In conclusion our results suggest the induction, by Fas-FasL, of a sort of trogocytosis and highlight a correlationship between trogocytosis and early apoptosis, not only for Fas-FasL induction but also for staurosporine treatment.

CIRCULATING TUMOR CELLS IN CANCER PATIENTS : METHODOLOGICAL CONTRIBUTIONS TO THEIR DETECTION AND IMMUNOMAGNETIC SEPARATION

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The separation and counting of a very low frequency of foreign cells, abnormally present in the peripheral blood, had greatly increased interest in the last decade and is now established as a "rare events" analysis. In particular, epithelial circulating (tumor) cells (CTCs) are frequently detected in the blood of cancer patients and their separation and count are important prognostic information and might help to tailor systemic therapies. Being the expected number of cells very low (1 to 100 in 10 ml of blood) the designed technical- methodological approach is crucial. Among the various available separation procedures we focused on those based on the immunomagnetic capture and in particular on a procedure originally developed and tested in our laboratory. The innovative step of the method is the possibility to perform the entire procedure in a multiwells plate and to directly observe and count the separated cells at the bottom of the wells. This is allowed by a dedicated magnetic plate fitting the shape of the disposable standard 8 multiwells plate.

As immuno-labelling capture procedure we focus on EpCam (CD326 Myltenyi 130-080-301) surface antigen very well established as the labelling of choice for the recognition of cancer cells in a large variety of solid tumours. The second labelling step had been performed by means of a Dynal magnetic beeds (Invitrogen Dynabeads Pan Mouse IgG cat 110.41) whose peculiar characteristic is to be directly visible by light microscopy. This feature had allowed to monitor both the labelling step as well as the final CTCs recovery by a simple staining with Propidium Iodide. Research is in progress aiming to a further biological/functional characterization of the separated cells.

CD133 AND CXCR4 EXPRESSION IN OVARIAN CANCER CELL LINEs

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Background: Ovarian cancer is the fifth leading cause of cancer deaths among women and the most common type of gynaecologic malignancy. Recent evidences suggest that neoplastic initiation and growth depend on a small subset of cells, termed cancer stem cells (CSCs). CD133 has been identified as a stem cell marker for normal and cancerous tissues, although its biological function remains unknown. A distinct subpopulation of $CD133^+CXCR4^+$cancer stem cells may play a role in the metastatic phenotype of the individual tumour.

Experimental Design: Ovarian cancer cell lines (OVCAR-3, OVACAR-4, OVCAR-5, OVCAR-8, IGROV-1, SKOV-3 and ADR-RES) and primary ovarian cancers were analyzed for CD133 and CXCR4 expression. Flow cytometry showed significative $CD133^+CXCR4^+$ cells in OVCAR-4 and OVCAR-5 cell lines; these data were confirmed by Western Blotting and immunocytochemistry. Sorted OVCAR-5/$CD133^+$ cells exhibited higher proliferation, self-renewal, colony-forming ability and forming sphere-clusters in serum-free medium with a high clonogenic efficiency in comparison with OVCAR-5/$CD133^-$. In addition it was possible to isolate the side population profile in $CD133^+$ and $CXCR4^+$ ovarian cell lines and expression of ABCG2 transporters. Furthermore, OVCAR-5/$CD133^+$ overexpressed CXCR4 compared to CD133 negative population. OVCAR-5/$CD133^+$ cells exhibit enhanced resistance to platinum-based therapy, drugs commonly used as first-line agents for the treatment of ovarian cancer.

Conclusions: We described $CD133^+CXCR4^+$ cells in ovarian cell lines and primary tumours. OVCAR-5/$CD133^+$ cells exhibit stem cell-like features such as high proliferation, self-renewal ability and are characterized by higher resistance to chemotherapy. Strategies aimed at modulating the SDF-1/CXCR4 axis may have important clinical applications to inhibit metastasis of cancer stem cells.

EGF DOWNREGULATES EXPRESSION OF CDC25A GENE IN BREAST CANCER CELL LINES

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Background: The phosphatase Cdc25A is a major regulator of both G1/S and G2/M transitions during cell cycle progression.

This role appears consistent with the high incidence of its misregulation in cancer; it has been shown that Cdc25A is overexpressed in primary breast tumors and this overexpression is correlated with an increased cell proliferation and with a poor prognosis in patients with breast cancer.

In a previous work the authors have suggested that EGF treatment induced a modest effect on cell proliferation and a transitional G1 arrest in MCF-7 cells.

To evaluate this hypothesis, aim of our study was to identify, through the analysis of gene expression, the main factors involved in this process of cell cycle slowing in breast cancer cell lines.

Methods: A microarray analysis, using Affymetrix GeneChip expression arrays, are performed inMCF-7 and SKBR3 breast cancer cell lines stimulated with epidermal growth factor (EGF), to compare the differential gene expression profile of breast cancer cells treated and untreated controls.

This analysis allowed us to obtain a statistically significant (p-value < 0.05) differential expression genes, and we selected a set of genes involved in cell cycle progression and tumor pathogenesis.

Results: We found a down-regulation of *CDC25A* and *E1,E2,D3 cyclins* genes, known to be involved in the G1 phase, both MCF-7 and SKBR3 breast cancer cell lines.

Focusing on *CDC25A* gene, we showed a reduction of mRNA levels and of related protein, by Real-Time RT-PCR and Western Blotting, with a greater reduction in the gene expression and protein levels, higher in MCF-7 cells.

Conclusions: These data suggest that EGF treatment induced a reduction of *CDC25A* expression and, as previously demonstrated, we hypothesize a temporary cell cycle arrest in the G1 phase, that seems to depend on this downregulation.

Therefore, if our results are confirmed by subsequent cytofluorimetric analysis, in the future phosphatase *CDC25A* could be an important therapeutic target in breast cancer and play a key role in the new therapeutic strategies.

CYTOMETRY AND DNA PLOIDY: CLINICAL USES AND MOLECULAR PERSPECTIVE

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Flow cytometry is one of the most powerful and specific methods for the integrated study of the molecular and morphological events occurring during cell proliferation, and many methods have been described for investigating this process. Many cell cycle regulators controlling the correct entry and progression through the cell cycle are altered in tumors. Infact, in most, if not all, human cancers show a deregulated control of G1 phase progression, a period when cells decide to start proliferation or to stay quiescent. Moreover, clinical studies focused now on the rapidly growth of tumor, determined by fraction of proliferant cancer cells relative to normal cells. Cytometry (flow and image) is capable to analyze DNA content thanks the use of same "molecule" conjugates with a fluorochrome that permits to identify DNA content of single cell in a sample. We have reviewed the most important results of studies on DNA ploidy during the last years. We have seen that analyses of DNA ploidy in cancer may provide clinically useful information on diagnostic, therapeutic and prognostic aspect. Infact, aneuploid cancer has a high proliferative activity and a metastatic or invasive potential, markers of a poor prognosis. Nowadays many proliferation markers and oncogene products have been discovered and their application in clinical oncology seems to be very promising. Multiparametric flow cytometry should allow the contemporaneous determination of morphology, phenotype, intracellular protein expression, and status of chromatin and of DNA. Evaluate if a particular protein is responsible of aggressiveness of cancer, or if it is responsible of alteration of DNA content, or if his activated state is the cause of quickly growth of cancer cells, is an important result that can help clinical approach to patients.

CANCER STEM CELLS-LIKE MARKERS IN SOLID TUMORS

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Cancer stem cell-like (CSCs-like) or initiating tumor cells, cell subpopulations with stem cells properties, was found in many solid tumors using cell surface markers and side-population by flow cytometry. Numerous putative markers are under investigation with the most significant body of work in brain, breast, colon, liver, lung, prostate cancer; to characterize and compare the specific markers that have been found to be present on CSCs is important for the future directions in this intriguing new research field. Moreover, surface markers were analized also in vitro tumor cells-sphere and in trsplantation experiments in NOD-SCID mouse and cells capable of forming sphere or tumor in vivo were characterized by the same specific markers. Some markers commonly used are CD34, CD133, CD24, and CD90, CD117, CD20, CD29, CD31 often in association; $CD44^+$, $CD24^{-/1ow}$, (with worse outcome: $CD44^+$, $CD24^-$, PROCR+), CD29, CD133+ in breast tumors, $CD133^+$ and CD166, CD44, CD49f, ESA in colorectal tumors, $CD133^+$, CD34, CD24 in lung tumors; in many case the tumors that formed from these cells recapitulated the histologic characteristics of primary tumors. Our data in solid tumors are concordant with the work that proposed

CD 133 as important marker in prostate, breast and lung tumors.

These markers have facilitated CSCs-like identification in multiple tumor sites and metastasis, but the impact of tissue digestion on marker specificity must be evaluated; moreover cell surface molecules analized by flow cytometry, may not ideal for histochemical analysis. Nevetherless, the explosion of new data, in this exciting field, but no single marker in any sites has emerged as definitive solution and many works support an important role of inhibitors of signaling pathways involved in self-renewal, growth and survival of these cells (Hedgehog, Wnt/B-catenin, Notch, ABC multidrug efflux transporters et al.). If indeed cancer stem cell-like are mediators of recurrence and metastasis, in fact several experimental data suggest that CSCs-like can be resistant to therapy, methods to identify these cells will represent a significant advance in cancer therapy with new strategies target signaling pathways that are involved in the self-renewal processes of CSCs.

ANALYSIS OF GERMLINE GENE COPY NUMBER VARIANTIONS IN PATIENTS WITH SPORADIC PANCREATIC ADENOCARCINOMA

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Background: The rapid fatality of pancreatic cancer is, in large part, the result of a diagnosis at an advanced stage in the majority of patients. Identification of individuals at risk of developing pancreatic adenocarcinoma would be useful to improve the prognosis of this disease. There is presently no biological or genetic indicator allowing detection of patients at risk of developing sporadic pancreatic cancer.

Methods: We analyzed gene copy number variations (CNVs) in leucocyte DNA from 31 patients (24 Europeans and 7 Japanese) with sporadic pancreatic adenocarcinoma and from 93 matched controls. Genotyping was performed with the use of the GeneChip Human Mapping 500K Array Set (Affymetrix). The HapMap database was used as the reference set.

Results: Our main goal was to identify CNVs common to all patients with sporadic pancreatic cancer. We identified 431 SNP probes with abnormal hybridization signal present in the DNA of all 31 patients. Of these SNP probes, 284 corresponded to 3 or more copies and 147 corresponded to 1 or 0 copies. Several cancer-associated genes such as CDC14B, CENPE, EIF2S2, FGF20, FZD10, GTF3C3, KLHL1, NOTCH3, RAB21, TULP3, VSNL1 and ZWINT were amplified in all patients. In addition, several genes supposed to oppose cancer development such as ASH1L, CD9, GRB14, IER3, LPXN, MAP3K7, MDC1, MINK1, SGPL1 and VRK1 were present as single copy in the genome of all 31 patients. Other genes involved in cancer such as BMP1, EGFL11, FLT4, FOSB, KIT, MAP4K4, MYB, PDGFRA, TGFA, AKT3 and KRAS were found amplified in almost all patients, whereas only one allele of the Myc inhibitor PAK2 and ARRB2 was detected in the majority of these patients. The set of the 431 SNP probes with abnormal hybridization signal of patients with sporadic pancreatic cancer was checked in the 93 control patients. None of them showed more than 5% match.

Conclusions: These data suggest that the set of 431 CNVs detected in the DNA of patients with sporadic pancreatic adenocarcinoma is associated to the disease. This CNV set could be used for early diagnosis of individuals with a genetic predisposition to develop a sporadic pancreatic cancer, for understanding the physiopathology of this disease and also to target these genes in a preventive strategy.

FUNCTIONAL ACTIVITY OF CXCL8 RECEPTORS, CXCR1 AND CXCR2, ON HUMAN MALIGNANT MELANOMA PROGRESSION

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We examined the autocrine/paracrine role of interleukin 8 (CXCL8) and the functional significance of CXCL8 receptors, CXCR1 and CXCR2, in human malignant melanoma proliferation, migration, invasion and angiogenesis. We found that a panel of seven cell lines, even though at different extent, secreted CXCL8 protein, and expressed CXCR1 and CXCR2 independently from the CXCL8 expression, but depending on the oxygen level. In fact, hypoxic exposure increases the expression of CXCR1 and CXCR2 receptors. The cell proliferation of both M20 and A375SM lines, expressing similar levels of both CXCR1 and CXCR2 but secreting low and high amounts of CXCL8, respectively, was significantly enhanced by CXCL8 exposure and reduced by CXCL8, CXCR1 and CXCR2 neutralizing antibodies, indicating the autocrine/paracrine role of CXCL8 in melanoma cell proliferation. Moreover, an increased invasion and migration in response to CXCL8 was observed in several cell lines, and a further enhancement evidenced under hypoxic conditions. A CXCL8-dependent *in vivo* vessel formation, evaluated through a matrigel assay, was also demonstrated. Furthermore, when neutralizing antibodies against CXCR1 or CXCR2 were used, only the involvement of CXCR2, but not CXCR1 was observed on cell migration and invasion, while both receptors played a role in angiogenesis.

In summary, our data demonstrate that CXCL8 induces cell proliferation and angiogenesis through both receptors and that CXCR2 plays an important role in regulating the CXCL8-mediated invasive and migratory behaviour of human melanoma cells. Thus, blocking the CXCL8

signalling axis promises an improvement for the therapy of cancer and, in particular, of metastatic melanoma.

EXTENDED CYTOMETRIC CHARACTERIZATION OF COLON CANCER STEM CELL SUBPOPULATIONS FROM FRESH TISSUE BIOPSIES: A PREREQUISITE FOR *IN VIVO* STUDIES

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The aim of this work was to characterize by flow cytometry colon carcinoma (CC) cells, identifying new specificities useful for Cancer Stem Cell (CSC) sub-setting. Although it is well accepted that colon CSCs do express CD133, this antigen identifies a heterogeneous population including, along with stem cells, more mature progenitor cells. We analyzed, by a panel of 29 antigens (CD133, CD9, CD24, CD26, CD29, CD44, CD47, CD49b, CD49f, CD54, CD55, CD56, CD59, CD66, CD66acd, CD66b, CD66c, CD81, CD90, CD112, CD151, CD164, CD165, CD166, CD200, CD221, CD227, CD324, CD326, β-catenin) cell suspensions derived from 10 fresh CC samples. The analysis was performed by a BD FACSAria equipped with four lasers (633nm, 488nm, 407nm and 375nm laser) using a set of 16 multiparametric combinations. We obtained a complex mosaic of cancer cell populations. We focused our attention on antigens present within the CD133+ population and useful for its sub-setting. CD55, CD66acd, CD66c and CD326 were expressed by the whole CD133+ population. By contrast, CD9, CD24, CD26, CD29, CD49b, CD49f, CD54, CD66b, CD90, CD151, CD164, CD227 were expressed with different intensities among CD133+ subset, thus allowing CD133+ population sub-setting. The most convincing and reproducible data were provided by CD227, CD151, CD90, CD164, CD26 and CD24. Interestingly, CD66c and CD55 resulted always over-expressed on CD133 positive cells as compared to CD133 negative cells. Some antigens, e.g. CD90, were not expressed in all analyzed tumors, possibly indicating a more aggressive phenotype. The development of new strategies for the correct analysis of CC tumor cell heterogeneity in fresh sample-derived cell suspensions is a fundamental step for analyzing the complexity of the stem cell compartment and it is a prerequisite for studies about the differential tumorigenic activity of selected subsets *in vivo*.

CHIKEN OVOALBUMIN UPSTERAM PROMOTER-TRANSCRIPTION FACTOR II (COUP-TFII) IN NORMAL AND HYPOPLASTIC HUMAN FOETAL LUNG

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COUP-TFII is an orphan member of the steroid receptor superfamily. It regulates the transcription of some genes involved in pulmonary development. Our aim was to clear up COUP-TFII localization in normal human foetal lungs to compare it with the one in the hypoplastic lungs of congenital diaphragmatic hernia (CDH) affected foetuses.

Thirteen normal and ten hypoplastic human foetal lungs, from 11 to 21 weeks of gestation, were studied. All foetal lung specimens were suitably treated for immunohistochemistry. Anti-COUP-TFII antibody was used to detect the protein. Anti-p63, anti-CD31, anti-Vimentin and anti-αSMA antibodies, were used to characterize COUP-TFII positive cells. Weigert staining was used for the same purpose. COUP-TFII nuclear immunoreactivity was observed, both in normal and pathological conditions, in mesenchymal cells of lung stroma, endothelial cells of lung veins, adventitia of lung arterioles and basal cells of the main bronchi, from 11 to 21 weeks of gestation. At 21 weeks a nuclear positivity appeared in smooth muscle precursor cells of lung arterioles. It is known that COUP-TFII is involved in muscle development and differentiation. Normal and pathological specimens showed a similar pattern of COUP-TFII distribution, but at 21 weeks of gestation hypoplastic lungs showed a greater number of COUP-TFII positive smooth muscle precursor cells in arterioles than normal foetal lungs. This may suggests that COUP-TFII could be responsible for the development of hyperplastic arterioles, which determine pulmonary hypertension, the major cause of death in CDH. We intend, therefore, to further investigate the role of COUP-TFII in arterioles development, both in normal and in hypoplastic human foetal lungs.

PROGNOSTIC VALUE OF FLOW CYTOMETRIC DNA PLOIDY IN COLORECTAL CANCER

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Several studies demonstrated that flow cytometric nuclear DNA content and Mismatch Repair (MMR) status are relevant prognostic factors in colorectal cancer (CRC). It has also been suggested that the prognostic value of DNA ploidy is mainly determined by its relationship with MMR status. Aim of the present study was to evaluate the prognostic significance of DNA ploidy in a large series of CRCs, characterized for several clinical and pathologic variables and MMR status.

The study included 415 patients with stage II and III CRCs surgically resected with curative intent. DNA ploidy was evaluated by flow cytometry using multiple frozen tumor samples. MMR status was determined by microsatellite analysis and/or immunohistochemical analysis of MMR proteins (MLH1, MSH2 and MSH6) expression.

296 (71.3%) tumors were classified as aneuploid and 119 as diploid. DNA ploidy was related to tumor site, tumor grade and type, aneuploidy being more often detected in carcinomas of the left colon and rectum ($P < 0.001$), well/moderately differentiated ($P = 0.002$), and in common adenocarcinomas ($P < 0.001$). Most tumors with deficient MMR were DNA diploid (62/75, 82.7%), whereas carcinomas with proficient MMR were frequently aneuploid (283/340, 83.2%; $P < 0.001$). Patients with diploid tumors showed more favourable clinical outcome than patients

with aneuploid tumors (P = 0.0007). This difference was also observed in the group of patients with proficient MMR carcinomas (P = 0.0006). In mutivariate survival analysis, TNM stage, grade of differentiation, MMR status and DNA ploidy were selected as independent prognostic variables.

Our results demonstrate that DNA ploidy is related to pathologic and molecular features in CRC and suggest that flow cytometric nuclear DNA content analysis provides prognostic information additional to MMR status.

LAMIN A/C AND N-MYC INTERPLAY IN NEUROBLASTOMA CELL DIFFERENTIATION

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Neuroblastoma is a childhood tumor of the peripheral sympathetic nervous system thought to arise from highly proliferative migratory cells of the neural crest. Amplification of the *MYCN* gene occurs in a number of neuroblastomas representing a hallmark of a highly aggressive subgroup. Most malignant neuroblastoma cells have retained their capacity to differentiate *in vitro*. The type-V intermediate-filament lamins A/C have an expression pattern in some organs dependent on the differentiation states. In adult tissues, lamin A/C is observed only in final differentiated cells. Our aim was to study the interplay between *MYCN* and *LMNA* genes in the neuronal differentiation. We employed the neuroblastoma cell lines SHSY5Y, with high lamin A/C and absent N-Myc expression, and LAN-5, with high N-Myc and low lamin A/C. Both cell lines were induced to differentiate by retinoic acid. Differentiated SHSY5Y cells reduced c-Myc protein expression, as expected and increased lamin A/C, suggesting an involvement of the lamins in the differentiation process. To better understand the role of lamin A/C in the SHSY5Y differentiation we silenced the *LMNA* gene. The decrease of lamin A/C determined an inhibition of the neurites formation, even though a decrease of the c-Myc protein was still evidenced. We then inhibited c-Myc protein activity by using a validated peptide which interferes with the Myc-Max dimerization. An increase of the neurofilament and of lamin A/C expressions was observed after exposure to the peptide, further indicating that lamin A/C is needed to differentiate SHSY5Y cells. By contrast, LAN-5 cells significantly increased N-Myc during their differentiation, while no increase of lamin A/C was observed, strongly indicating an interplay between *NMYC* and *LMNA* genes.

CIRCULATING ENDOTHELIAL CELLS IN METASTATIC COLORECTAL CANCER: POTENTIAL BIOMARKERS OF RESPONSE AND RESISTANCE TO ANTIANGIOGENETIC THERAPY

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Background: Little progress has been made in the validation of prognostic and predictive biomarkers for selecting pts with cancer for antiangiogenic therapy. Circulating endothelial cells (CECs) and their progenitors (CEPs) number and function by FCM analysis has been proposed as non- invasive biomarkers of efficacy. To further confirm this hypothesis, we now focused on the prospective modification of CECs and CEPs during a Bevacizumab - based therapy in advanced colorectal cancer (mCRC) pts.

Methods: We evaluated CEC (resting, rCECs: CD45-, CD106-, CD34+, CD146+ and activated, aCECs: CD45-, CD34+, CD146+,CD106+) and CEP (CD133+) absolute number by high-resolution FCM in 48 mCRC pts, at baseline and at the moment of the response evaluation. Data from a group of 50 healthy subjects (HS) were utilized as control.

Results: We observed: 10 complete responses (CR), 26 partial responses (PR), 6 stable diseases (SD) and 6 progressive diseases (PD). At baseline, with respect to HS, responder (CR+PR) pts showed an higher value of total CECs vs SD. The pts who obtained a clinical benefit (CR+PR+SD) showed a statistically significant decrease of total CECs (baseline vs response evaluation) and this difference became even more evident for CR+PR pts. The final value of the aCEC subset was also found to be higher in pts who obtained SD and in PD vs CR+PR. Finally, a statistically higher baseline level of CEPs was found in CR+PR (and in pts who showed a SD) with respect to HS.

Conclusions: Our data demonstrate that the kinetic of CECs and CEPs after a treatment with Bevacizumab + chemotherapy can be utilized to early identify pts who will obtain an objective response, thus confirming the clinical role of endothelial cells as biomarkers of tumor shrinkage.

ROLE OF VAV1 IN DIFFERENTIATION ALONG THE MONOCYTIC-MACROPHAGIC LINEAGE OF TUMORAL MYELOID PRECURSORS

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The signal transducer Vav1 is physiologically expressed only in hematopoietic cells. While in lymphocytes Vav1 appears crucial for both development and functions, in myeloid cells its role is restricted to the response of mature cells to external stimuli.

Our research group has identified Vav1 as a crucial molecule in the ATRA-dependent overcoming of the differentiation blockade along the granulocytic lineage of cells derived from acute promyelocytic leukemia (APL), the M3 subtype of acute myeloid leukemia (AML). In particular, by modulating its expression during ATRA-treatment, we have demonstrated that Vav1 is required for acquirement of a neutrophil-like differentiated phenotype by tumoral promyelocytes, in terms of CD11b expression and cell/ nuclear morphology.

Starting form these evidence, this work was aimed to establish whether Vav1 is also involved in the maturation of

tumoral promyelocytes along the monocytic-macrophagic lineage.

For this purpose, AML-M2- and AML-M3-derived cells were induced to differentiate to monocytes/macrophages by treatment with different agonists (ATRA, PMA, Vitamin D3), highlighting an increased Vav1 expression that paralleled the reached maturation level. The down-modulation of Vav1, during the drug treatment, obtained by means of specific siRNAs, negatively affected the differentiation process in terms of CD11b expression, adhesion capability and morphological changes. The role of Vav1 in modulating the maturation process and, in particular, the acquisition of a differentiated morphology, was more evident in AML-derived cells that reached higher levels of phenotypical differentiation (macrophage-like). The reported data indicate that, at variance with maturation of normal myeloid precursors, in which it seems to be ineffective, Vav1 plays a role in the progression of tumoral promyelocytes along the monocytic-macrophagic lineage, suggesting that this molecule is a potential target for the therapeutic treatment of acute myeloid leukemias.

PHOSPHATIDYLCHOLINE-SPECIFIC PHOSPHOLIPASE C AS A POSSIBLE TARGET IN BREAST CANCER THERAPY

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HER2 is a cell-surface protein with tyrosine kinase activity that is involved in the growth and differentiation of cells and has been detected in about 30% of breast carcinomas. This molecule is today considered as a therapeutic target through the induction of its down-regulation with consequent effects on cell proliferation and differentiation. Our recent studies showed a relationship between the higher expression levels of the phosphatidylcholine-specific phospholipase C (PC-PLC) on the membrane surface and the tumor progression in ovarian cancer cells, and pointed out the role of this enzyme in regulating CD16 membrane expression in Natural Killer cells.

In this work, we showed that PC-PLC is differently distributed on the plasma membrane of breast cancer cells and it is associated with HER2 in lipid rafts. The PC-PLC inhibition in HER2 over-expressing breast carcinoma cells induced a complete internalization of HER2 within 24 h, with a strong retardation of its re-expression on plasma membrane, and a deep impact on cell proliferation, inducing a block in cell cycle. Moreover, we showed that PC-PLC activity increased in all the tumor cell lines analyzed if compared with non tumoral cells and, in particular, among tumor cells the enzyme had the highest rate of activity in the highly metastatic cell line. Besides, we evaluated the effects of PC-PLC inhibition on lipid droplets induction and on the expression of the epithelial-mesenchimal transition (EMT) typical markers.

Altogether, these data suggest that PC-PLC enzyme could play an important role in regulating both tumour transformation and HER2 endocytic pathway. In fact, the inhibition of PC-PLC leads to the expression of typical EMT markers and to the weakening of the oncogenic signal HER2-mediated, thus suggesting therapeutic strategies based on PC-PLC as a new molecular target in breast carcinoma.

DISSEMINATED TUMOUR CELLS IN THE TUMOUR DRAINING VEIN OF PATIENTS WITH NON-SMALL CELL LUNG CANCER

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Purpose: The aim of this study is to examine whether surgical manoeuvre or resection of lung cancer could lead to haematogenous dissemination of malignant cells in patients with non–small-cell lung cancer (NSCLC). Disseminated tumor cells in draining vein can be associated with an increased early recurrence.

Methods: Thirthy-three patients with completely resected primary non-small cell lung cancer (pT1-4 pN0-2) were admitted to the study. The blood samples obtained from pulmonary veins from the lobectomy or pneumonectomy were examined for occult tumour cells by immunocytochemical staining of cytospins using the pancytokeratin antibody A45-B/B3 that binds to the cytokeratins 8, 18 and 19.

Results: Disseminated cancer cells in pulmonary venous blood were observed in 7 out of 33 patients (21%).We could not find a statistically significant correlation with standard clinical-pathological parameters. However, we found that the incidence of disseminated tumour cells in pulmonary venous blood was different in patients with smaller size (pT1-pT2) in comparison to large extensions (pT3-pT4), respectively 17,2% versus 50%. All the patients with N2-limph node involvement were positive for the presence of disseminated cancer cells in venous blood. Moreover, we observed that the incidence of haematogenous dissemination of malignant cells was 41,6% in patients affected by squamous cells carcinoma, 33% in patients with large cells carcinoma, and, only 8,3% in patients with adenocarcinoma. No evidence of venous dissemination was found in patients affected by the other histologycal tumor types.

Conclusion: This study shows that occult disseminated cancer cells in the pulmonary venous blood are detectable in the 21% of the patients with resectable non-small cell lung cancer. The detection of such cells might be useful for the identification of patients who may benefit from adjuvant therapy.

IN VITRO AND IN VIVO FUNCTIONAL CHARACTERIZATION OF NEW CYCLE-PEPTIDES INHIBITORS FOR C-X-C CHEMOKINE RECEPTOR-4 (CXCR4)

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The C-X-C chemokine receptor-4 (CXCR4) is a receptor for stromal cell-derived factor 1α (SDF-1a/CXCL12) mainly implicated in lymphocytes homing. CXCR4 is also overexpressed in human cancers while SDF-1 α is preferentially expressed in organs sites of metastasis. Thus, efficient CXCR4 antagonists could are welcome to inhibit metastatic spreading. Through rationale design a new library (20 units) of cycle-peptide molecules, that consists of 5 and 7 amino-acid residues cycled by a S-S bridge, was generated based on SDF-1 α and v-MIP II analogy. CCRF-CEM, T-leukemia cell lines and PES43, human melanoma cell line, overexpressing CXCR4 were evaluated for the capability of CXCR4 inhibition through the above peptides. Indirect receptor binding and calcium flux were evaluated by flow cytometry, ERK1 and ERK2 phosphorylation, and cell migration were evaluated too. Four cycle-peptides showed a significant inhibitory activity on chemokine-induced receptor's activation. Supported by in vitro results we move to in vivo experiments. Treatment of CXCR4-B16 transduced mice showed inhibition of lung metastases in mice treated with 3 out of 4 peptides as compared to AMD3100. Ongoing experiments are evaluating the binding to the receptor in CCRF-CEM of a labelled SDF-1alpha (alexaFluor 647 labelled) to evaluate the specific binding and the effect of the inhibitory peptides. Thus according to our results cycled peptides CXCR4 inhibitors could play an important role as therapeutic agents against cancer progression.

CHARACTERIZATION OF MARKERS ASSOCIATED WITH TUMORIGENICITY IN OVARIAN CANCER TUMORS

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Ovarian ranks fifth in both cancer incidence and mortality in Western women and has a high mortality rate. Despite the fact that more than 70% of the patients respond to front line therapy (surgery followed by a platinum based chemotherapy) most of them will eventually relapse and die from chemo-resistant disease. As for other tumors, also for ovarian cancer tumor experimental evidences have been put forward on the existance of a tumor initiating cell. The aim of the present work was to try to identify and characterise the initiating cell from ovarian cancer. Markers reported to be associated with staminality/tumorigenicity in different solid tumours have been studied both in stabilised ovarian cancer cell lines and in fresh tumour ovarian samples. Specifically, we focused on the Side Population (SP) phenotype, the positivity for Aldehyde Dehydrogenase (ADH), positivity for CD133 and the capacity to form spheroids when cultured in growth condition selective for self-renewing. The SP phenotype was present in 37% of the fresh samples analyzed, with values ranging from 0.25 al 4.57 %; ADH positivity ranged from 0.2-46.8% in all tumor samples analyzed while CD133+ cells were found in the 50% of samples, with range values from 0.2-34%. Whenever possile, cells positive and negative for these markers were sorted and studied for their ability to grow in nude mice and cultured *in vitro*. Spheres could be obtained from different fresh samples and could passaged *in vitro* for different passages. Studies are ongoing to charatcerize these spheroids from a molecular, pharmacological and phenotypical points as well as for their ability to grow *in vivo*.

MTOR INHIBITION MODULATES THE BIOLOGIC PHENOTYPE OF CD24+CD133+ RENAL MULTIPOTENT PROGENITORS (RMP)

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Recent studies have described a population of Renal Multipotent Progenitors (RMP) in adult human kidneys at the urinary pole of the Bowman capsule, which are CD24 and CD133 positive and are able to repair injured renal tissue of SCID mice with glycerol-induced acute tubular necrosis (ATN) and adriamycin-induced nephropathy. mTor inhibitors (Rapamycin) may affect the RMP biology both *in vivo* and *in vitro* via the modulation of HIF1alpha/VHL pathway, which regulates downstream the CXCR4 and SDF-1 genes, both required for therapeutic homing of RMP in vivo. In this study we aimed to assess the effects of mTOR inhibition on the RMP phenotype proliferation, viability and chemotaxis.

RMP cell lines were isolated from normal kidneys of 30 patients undergoing nephrectomy for renal carcinoma. At first passage after isolation, flow cytometric analysis showed that the cell pool was CD24+ (60,0%) and CD133+ (25,4%), while only 20.6% of pool cell co-expressed both markers. Cell proliferation induced downregulation of these markers. After immunomagnetic enrichment CD133+ cells were doubled (25,4% vs 54,8%), while CD133+CD24+ cell significantly increased (13,7% vs 21,6%). Rapamycin treatment at different concentrations increased CD133+CD24+ cell population (17,8% vs 55,5% at 5 ng/mL vs 49,3% at 20 ng/mL), while it decreased RMP proliferation (-34,3% at 5 ng/mL and -43,3% at 20 ng/mL after 120 hours). Moreover Rapamycin treatment didn't affect cell viability (89,6% vs 91,0% vs 92,5%), while it didn't protect from apoptosis induced by H_2O_2 2mM pre-treatment for 18 hours (86,3% vs 87,1% vs 75,9%). Finally Rapamycin inhibited RMP chemokinesis (-37,6% at 5 ng/mL and -47,4% at 20 ng/mL after 24 hours).

These data suggest that in basal conditions Rapamycin treatment inhibits RMP proliferation and chemokines, while it doesn't affect RMP phenotype and viability, thus providing useful informations about RMP biology in the attempt to develop new therapies for acute and chronic renal injury.

FLOW CYTOMETRIC ANALYSIS OF LYMPHOCYTE AND DENDRITIC CELL SUBSETS IN METASTATIC COLORECTAL CANCER PATIENTS TREATED WITH CETUXIMAB- BASED THERAPY

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Background – Cetuximab, a chimeric immunoglobulin monoclonal antibody, targeted against the epidermal growth factor receptor (EGFR) was found to exert antibody dependent cellular cytotoxicity in several tumor cell lines. Multiple links have been found between the EGFR signalling pathway and the immune response in human tumors.

In order to inquire into the immunological mechanisms of action of Cetuximab we have focused on its in vivo impact on both the peripheral blood lymphocyte and dendritic cell (DC) immunophenotype in metastatic colorectal cancer (mCRC) pts.

Methods - The peripheral blood lymphocytes and DC subsets we analyzed by multicolor FCM in 18 pts (M/F:12/6, median age: 63 yrs; range 43-78) treated with Cetuximab-based therapy, in absence of clinically relevant infections. Baseline data were compared with reference values obtained by 50 healthy subjects (M/F: 25/25, median age: 43 yrs, range 21-65).

Results - With respect to normal donors in our pts at baseline we observed a significant lower level of the absolute lymphocyte number ($p = 0.0001$), B cells ($p = 0.0002$), T cells (0.001), NK cells ($p = 0.04$) and DCs with their subsets ($p = 0.0002$; $p = 0.002$; $p = 0.001$), while activated T cells showed a higher level ($p = 0.03$). After 3 courses of chemotherapy + Cetuximab, a trend was shown toward a progressive increase of all the lymphocyte subsets, of total DCs and of their subsets. This trend was confirmed after 6, 9 courses and at the time of disease evaluation.

Conclusions - These data show that Cetuximab seems to improve in vivo the T-cell mediated immune response in pre-treated in mCRC pts. This provides new insight into its possible additional antitumor mechanism and may be helpful in the design of combination therapy for mCRC pts.

EFFECTS OF CIGLITAZONE, PPARγ AGONIST, ON LEPTIN EXPRESSION IN MCF-7 AND MDA-MB-231 BREAST CANCER CELLS

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Background: Leptin, a hormone produced mostly by the adipose tissue, in addition to its well-documented role in the control of appetite and energy homeostasis, is known to regulate various physiological and pathological processes in the peripheral organs. Of note is the accumulating evidence that leptin can induce growth and progression of different cancer types and data obtained in cellular and animal cancer models demonstrated that can act as a mitogen as well as antiapoptotic transforming and motogenic factor.

The importance of leptin signaling in breast tumorigenesis has been confirmed by the fact that breast tumors overexpress both leptin and its receptor, both of which correlate with higher tumor grade and worse prognosis. In vitro studies demonstrated that breast cancer cells are able to synthesize leptin in response to obesity-related stimuli, like hyperinsulinemia and hypoxia. This process is mediated through interactions of Sp-1, a nuclear factor that mediates the effects of insulin and/or HIF-1, the master transcription factor in cellular response to oxygen deficiency, with specific motifs within the leptin gene promoter.

Considering that in adipocytes leptin promoter is regulated by the activation of peroxisome proliferator activated receptor (PPAR) γ, we studied whether or not ciglitazone, a PPAR-γ ligand, used for treatment of patients with diabetes and obesity and a potential anti-neoplastic agent, can modulate the expression of leptin mRNA in breast cancer cells.

Methods and results: Using chromatin immunoprecipitation (ChIP), we found that treatment of MCF-7 and MDA-MB-231 breast cancer cells with submolar concentrations of ciglitazone induced binding of PPAR-γ to the proximal portion of the leptin promoter, while it decreased the association of Sp-1 with this DNA region. Results obtained with Real Time PCR, Western blotting as well as growth experiments confirmed that these effects coincided with elevated leptin mRNA expression, protein synthesis and increased cell proliferation. The mitogenic effects of ciglitazone were not observed when higher doses of the drug were used.

Conclusions: These data suggest that one of the mechanisms of leptin overexpression in breast tumors might involve activation of PPAR-γ with submolar concentrations of ciglitazone.

IN VIVO TUMOR TARGETING BY IDIOTYPE-SPECIFIC PEPTIDES

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In order to limit the adverse side effects of cancer therapy, it is necessary to design new strategies of drugs delivery into tumor cells. Peptides are promising tools to deliver

radionuclides or therapeutic drugs to tumor cells. The idiotypic determinants of surface immunoglobulins of neoplastic B cells have unique amino acid sequences and can be regarded as highly specific tumor markers. This suggests that idiotype-specific peptides (Id-peptides) for the BCR (B cell receptor) of neoplastic B cells could target selectively transformed B cells. In this work, we evaluated the ability of Id-peptides for B-lymphoma cells selected by screening Random Peptide Libraries (RPLs) as a tool for the specific delivery of a therapeutic cargo into tumor cell. Results can be summarized as follows:

a) We selected three phage clones by screening three distinct RPLs with immunoglobulins purified from the murine B lymphoma cell line A20;
b) Synthetic peptides, corresponding to the insert of phage clones, maintained their antigenic properties;
c) Id-peptides were internalized into target tumor cells by BCR-mediated endocytosis;
d) When inoculated in tumor-bearing mice, Id-peptides targeted specifically tumor cells;
e) Id-peptides were able to specifically deliver a cargo protein (GFP) or radionuclides into target tumor cells both *in vitro* and *in vivo*.

These results show that Id-peptides are powerful tools for *in vivo* targeting of tumorigenic B cell lymphoma and to deliver therapeutic drugs selectively into tumor cells.

CHARACTERIZATION OF A HUMAN MYXOID LIPOSARCOMA (MLS) CELL LINE MADE RESISTANT TO TRABECTEDIN (ET-743)

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Recent clinical data have shown that the myxoid liposarcomas (MLS), whose pathogenesis is related to the expression of FUS-CHOP fusion gene, are extremely sensitive to T with prolonged remission in a high proportion of patients. Some patients are or become resistant to T and thus it is important to elucidate the mechanisms of resistance to T. We developed a resistant subline (402-91 ET/Res), by exposing MLS 402-91 cell line to increasing concentrations of T. 402-91 ET/Res cells mantained the expression of the FUS-CHOP chimera. The resistance to T was not related to an increase in Mdr related proteins and to a decreased intracellular drug retention. The resistant cells were cross resistant to Melphalan, while were more sensitive to Taxanes, Vinblastine, Temozolomide and UV rays. T induced a G_2M block only in the parental cell line, suggesting a different DNA repair mechanism in the 402-91 ET/Res cells. The collateral sensitivity of 402-91 ET/Res to UV rays suggested a possible impairment of NER function, that was in fact demonstrated. We found absence of XPG gene and protein, in resistant cells. These results further stress the relevance of NER mechanisms in the mode of action of T, and highlight the need of further studies to evaluate this mechanism in clinical samples.

MICROSCOPIC CHARACTERIZATION OF IN VITRO AND IN VIVO PRECLINICAL MODELS OF COLORECTAL CANCER

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Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the western world. The metastatic disease occurs in 35–50% of patients, and once metastases have developed, the prognosis is often poor. Although advances in radiotherapy, chemotherapy, and immunotherapy, surgical excision of the localized disease is currently the only means of improving patient survival. In this context, preclinical models of primary and metastatic CRC appear very useful for discovering novel tumour biomarker and testing innovate preventive/therapeutic strategies. To these purposes, in our laboratory we characterized *in vitro* and *in vivo* preclinical models of CRC.

In vitro model: DHD/K12 cell line, originally established from 1,2-dimethylhydrazine(DMH)-induced CRC in BDIX rats, displaying many of the features of epithelial cells constituting the human CRC tissue (expression of tumor-associated antigens, some differentiation markers and crucial molecules of pathways involved in the carcinogenetic process).

In vivo models of primary and metastatic CRC in syngeneic immunocompetent BDIX rats: 1) DMH-induced CRC model, useful for studying early carcinogenesis and sporadic cancer development, in which we determined the timing of sequential steps of carcinogenetic process and analyzed changes in the expression of some crucial molecules of pathways involved in CRC insurgence and progression. 2) Syngeneic models, in which cancer is induced by injecting DHD/K12 cells in different sites, including: 2a) Subcutaneous injection in the shaved cervical region (subcutaneous tumor); 2b) Intraperitoneal injection (peritoneal carcinosis); 2c) Simultaneous injection of cells in the splenic vein and in the inferior vena cava (synchronous liver and lung metastases). The last two models mimic the clinical situation of metastatization after primary tumor surgical resection, with high biological and immunological affinity to human neoplasia.