SOCIETÀ ITALIANA DI CITOMETRIA

# PROCEEDINGS OF THE XXIX NATIONAL CONFERENCE OF CYTOMETRY

## *Centro Congressi Grand Hotel Salerno*

**Salerno**
**Italy October 5—8, 2011**

EDITED BY
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# XXIX National Conference of the Italian Society of Cytometry GIC

## October 5—8, 2011
## Salerno, Italy

Based on the previous fruitful experiences also this year an issue of Cytometry will be partly dedicated to the program and abstracts of the National Conference of the Italian Society of Cytometry, GIC. The XXIX edition of the Conference had been held in Salerno City, Italy from October 5$^{th}$ to 8$^{th}$. Salerno is located on the Gulf of Salerno on the Tyrrhenian Sea, it is the main town close to the wonderful Costiera Amalfitana (including the famous towns of Amalfi, Positano, and others). One of the first University of Medicine in the world was funded in Salerno (*Schola Medica Salernitana*). During the 16th century the city became a great centre of learning, culture and the arts.

As far as the GIC meeting is concerned, we would like to stress the fact that all abstracts were carefully reviewed by the Scientific program Committee and published here in full and categorized by scientific tracks. 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 the Scientific Committee selected 124 abstracts among those submitted by several Italian research teams. Each session involved invited lectures and was focused on the emerging role of cytometric techniques in Hematology, Stem Cell Biology, Immunology, Oncology and Environmental Sciences and Toxicology.

In addition, different topics of general interest have been presented during the plenary sessions, in particular we offered two special lectures on the recent advances of flow cytometry in the fields of vaccines and pediatric oncohematology.

The Conference was characterized by special symposium on the role of minimal residual disease in oncohematology in which four experts in the field have been invited to bring their top contribution. A session was devoted to the preparation of guidelines for the immunophenotyping of leukemias and related fields. A special lecture also emphasized the accreditation and certification role in flow cytometry.

Moreover, as traditionally promoted by GIC, a round table among several Italian Scientific Societies, having different levels of interest in cytometric applications was organized in order to promote interactions and collaboration programs.

A substantial contribution has been provided by the principal companies in the field, located in a large exhibition area inside the conference center. This is the largest national event in cytometry representing the greater Italian cytometry's scientific contribution to the international community.

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# TABLE OF CONTENTS

## INVITED SPEAKERS

### STEM CELLS IN SOLID TUMORS: FROM BENCH TO BEDSIDE

**De Maria R.**
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The existence of cancer cells with stem cell-like features has been documented for the first time in leukemia and, more recently, in a series of solid tumors. It is now accepted that this rare subpopulation of tumor cells, called cancer stem cells (CSCs), is responsible for the onset, maintenance, and possibly relapse of the tumor in patients previously treated with chemotherapy or radio-therapy. Thus, these cells should be the preferred target of effective therapies aimed to eradicate the tumor. The development of technologies that allow the unlimited *in vitro* expansion of CSCs represents a powerful tool for the study of pathogenic events that cause the initiation and progression of the tumor both for screening and pre-clinical development of novel therapeutic compounds. In recent years our group has developed a technology that allows the isolation and *in vitro* expansion of CSC from several solid tumors, including glioblastoma, melanoma, breast, lung, colon, thyroid and ovarian cancer. We are currently characterizing these populations of cancer cells at different levels, including gene expression profiling and proteomic analysis of microRNA. The extensive molecular characterization may allow the identification of markers more specific for the CSCs, providing important information on potential drug targets relevant to develop a more effective strategy of target therapy. In addition, the use of CSC to generate xenografts that faithfully reproduce the patients' tumors, as assessed by morphological and molecular analysis, offers a unique opportunity to test new cancer treatments and, potentially, to optimize individual therapy.

Therefore, although the identification of the CSC is relatively recent, this research looks extremely promising for potential clinical development. It's likely that understanding of the molecular mechanisms underlying tumorigenesis and aberrant survival of CSCs can significantly contribute to the rational design of new targeted cancer therapies.

### CLINICAL EFFICACY OF CELLULAR TERAPIES: A CRITICAL APPROACH

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Somatic advanced cell therapy products (ATMP) are emerging as innovative therapeutic possibilities in several different fields of medicine. In previous years, nonetheless, a lot of experimental data on in vitro or animal models have created expectations as to regenerative capacity of different kinds of stem cells and even contributed to the emergence of complex hypotheses of dedifferentiation, transdifferentiation and yet others. These results and their interpretations have reached general public and the press, creating a climate of expectations and also the basis of an ever growing profit-based market including badly identified clinical structures all over the world which were claimed able to offer every sort of therapy for the most devastating human diseases based on unidentified "stem cells".

Scientific rigorous approach is badly needed to explain to the public what the really available therapies are present today, with which stem cell type and for which disorders.

Clinical solid data have to be shown and discussed with transparent attitude and possibly no conflict of interest. In particular we will show our data with hematopoietic stem cells from bone marrow, peripheral blood or cord blood and with mesenchymal stromal cells. Moreover data with epithelial cells will also be shown and discussed.

Finally we will remember which are the rules and the standards necessary today in Italy to perform ATMP clinical trials with the approval of all the Competent Authorities involved.

### THE NEW FLOW CYTOMETRY IN MYELODYSPLASTIC SINDROMES (MDS): FROM CD34+ PRECURSORS TO PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) CLONES

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Clonal transformation in MDS occurs in a CD34+ myeloid committed stem cell. Interesting results in the perspective of a diagnostic use of FCM in MDS patients derive from the analysis of immunophenotypic abnormalities of CD34+ cells. The vast majority of CD34+ cells in MDS are committed to myeloid lineage (CD38+HLA-DR+CD13+CD33+) and the decrease of hematogones is one of most consistent findings in MDS patients. The evaluation of percent CD34+ B-cell progenitors has negligible inter-operator variability and seems to be reliable in many centers. Asynchronous co-expression of stemcell and late-stage myeloid antigens (CD117/CD15, or CD117/CD11b) or abnormal expression of lymphoid markers (CD56, CD2, CD5, CD7, CD19) have been reported. It has been also evaluated the expression of CD38 on CD34+ cells as a diagnostic tool in MDS. B-cell progenitors highly express CD38, and low numbers of B-cell progenitors in MDS can reduce the mean fluorescence

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intensity of CD38. The CD38 display on CD34+ cells can be be further reduced in MDS since in high risk patients there are elevated numbers of immature CD34+CD38-progenitors.

All these findings strongly suggest that CD34-related parameters are good candidates as diagnostic markers in MDS. The evaluation of CD34-related parameters could to be useful in patients without specific markers of marrow dysplasia (ring sideroblasts, chromosomal abnormalities). During last years important progresses in the understanding the meaning of PNH clones in MDS have been obtained. Full understanding of PNH will have larger implications than for PNH itself, as indicated by the therapeutic implications of the PNH clone presence in MDS: it could disclose an immunological pathogenesis for low risk MDS, such suggesting an immune system targeted therapy.

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FISH LABELLING IN SUSPENSION (ISFISH) AND FLOW CYTOMETRY: NEW ACHIEVEMENTS AND APPLICATIONS

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Flow sorting is always been rewarded as a very interesting and useful application from the beginning of flow cytometry. Among others, chromosome sorting is one of its remarkable achievements, but useful flow cytometry specific chromosome stains are very limited and a true chromosome discrimination can be only made on the basis of total DNA amount and/or AT/CG relative content. In cytogenetic, fluorescence *in situ* hybridization (FISH) is an extremely powerful methodology allowing a fine discrimination and identification of single chromosome types and most of their rearrangements up to single gene localization. Many unsuccessful attempts have been carried on to combine the good of both techniques to develop a whole and consistent system for high definition sorting of chromosomes in suspension, with the only exception of interphase nuclei. Labelling chromosomes in suspension with specific probes could help in isolating normal and mutant chromosomes for a number of molecular manipulations both in humans and plants. We focus our research on developing a rapid, reproducible and affordable strategy for *in situ* fluorescence labelling of in suspension chromosomes (ISFISH). This new procedure allows flow karyotyping and quantitative sorting of single type chromosomes from standard genotypes where chromosomes cannot be identified by DNA content variations. We developed ISFISH on plant chromosomes with the aim to sort large numbers of chromosomes from top quality bread and pasta wheat (*Triticum durum* and *T. aestivum*) varieties where genes for quality production, for biotic and abiotic stresses and also for good agronomic performances are present, thus avoiding the use of mutant lines (such as substitution or addition lines) usually breeded from poor wheat materials. We checked and used several probes capable to generate reproducible highly informative DNA banding patterns. Chromosome karyotyping and flow sorting was performed on the basis of DNA content and probe labelling. Samples can be prepared in 60 min about, using 50 ng probe/106 chromosomes. ISFISH works equally well on squashes and banding patterns showed to be true-to-type in respect to classic FISH method. This technique allowed detecting and sorting a single chromosome type in commercial wheat genotypes and all the chromosome types in wild grain species (*D. villosum*). In our opinion, ISFISH opens new avenues in chromosome flow sorting and its medical and biotech applications.

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THE USE OF FLOW CYTOMETRY FOR THE DETECTION OF ENVIRONMENTAL BACTERIA IN DIFFERENT ECOSYSTEMS

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Flow Cytometry is a powerful tool for the analyses in environmental microbiology. FCM can provide a broad range of information at the single-cell level, including total counts, size measurements, nucleic acid content, membrane potential, cell viability and activity.

Furthermore, the simultaneous use of FCM with specific antibodies permits the detection of bacterial groups or species, increasing the knowledge of microbial community also in marine and in freshwater ecosystems. Data from the application of a polyclonal antibody for the detection of *V. parahaemolyticus* in pure culture and seawater are presented.

The combination of cytometric rapidity and multi-parametric accuracy with the phylogenetic specificity of oligonucleotide FISH probes has been regarded as a powerful and emerging approach in aquatic microbiology. Nevertheless, few technical improvements have been proposed in the last 5 years. Recent attempts and efforts on environmental samples analyses are reported to remark the importance and the potentiality in microbial ecology of combining CARD-FISH and Flow Cytometry.

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APPLICATION OF FLOW CYTOMETRY, ANALYTICAL CYTOLOGY AND IMMUNOHISTOCHEMISTRY IN THE CHARACTERIZATION OF IONIZING RADIATION EFFECTS ON MALE GERM CELLS

**Cordelli E., Bartoleschi C., Benassi B., Eleuteri P., Grollino M.G., Pardini M.C., Pacchierotti F., Spanò M. and Villani P.**
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Sperm DNA integrity is essential for the accurate transmission of paternal genetic information. Spermatozoa are the final result of a complex and ordered differentiation process taking place in the testis. The testis, beside being one of the most radiosensitive organs, has a variety of cells which differ in their degree of radiosensitivity. This different sensitivity depends on the differentiation stage, chromatin condensation, nucleoprotein composition and repair capability. Due to the complexity of spermatogenesis and the heterogeneity of testicular cell subpopulations, an accurate characterization of radio-induced damage in this tissue is difficult and requires a multidisciplinary approach which allows the identification of damage in the different cellular compartments.

We applied neutral comet assay, immunodetection of phosphorylated histone H2AX (γ-H2AX) and sperm chromatin structure assay (SCSA) to detect the production of DNA strand breaks in testicular cells and spermatozoa at different times after in vivo X-ray irradiation. Results show that irradiation of testicular cells induced DNA strand breaks which were repaired within a short time. We also found that the kinetics of H2AX phosphorylation -dephosphorylation was not linked with the DNA strand breaks removal since γ-H2AX foci persisted when DNA damage was no more detectable by comet assay. Spermatozoa were resistant to the induction of DNA damage, but nontargeted DNA breaks were found in spermatozoa deriving from radiation-targeted proliferating spermatogonia, becoming evident when the spermatids started to elongate in testicular seminiferous tubules. We also show that, at that time, transcription of pro-apoptotic genes was enhanced, suggesting that an apoptotic-like process could be involved in the DNA breaks formation.

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## FLOW CYTOMETRY, IMMUNOHISTOCHEMISTRY AND QUANTITATIVE mRNA EXPRESSION: COMPARATIVE APPROACHES FOR DETECTION OF CD133+ CANCER STEM CELLS IN TUMOURS

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Background: CD133 marker is the main marker for isolation of cancer stem cells (CSCs). Unfortunately, its correct determination on cellular surface can be prejudiced by several factors. Here, we analyze CD133 expression in breast cancer by cytometric analysis, immunohistochemistry and Quantitative Real Time PCR and we evaluated if three methodologies are efficacy in detecting of CD133 marker.

Methods: In this study, 12 patients were enrolled at the National Cancer Institute of Naples. For each patient, immunohistochemical staining for CD133, E-cad, CD44 markers was performed on embedded paraffin tissues. Real Time-PCR for CD133 on frozen biopsies was also performed. Moreover, the tissue obtained from surgery was tested by flow cytometry for CD44 FITC, CD326 PE, CD133PE and CD45APC.

Results: By immunohistochemistry, the E-cad was expressed in 11 cases. The percentage of CD133+ cells was very heterogeneous. In 5 samples, the expression was very low or absent, in 5 it was moderate (5–15%), in one case it was >20% and only in Tubulobular variant breast cancer, it was about 35%. CD44 expression was also extremely variable. By flow cytometry, the mean percentage of CD133 positive cells was 13,75% and the mean percentage of CD44 positive cells was 17,90%. All cells analyzed were positive for CD326 (EpCAM). In Tubulobular breast variant, the percentage of CD133 was about 70%. Both immunohistochemistry and cytometric data for CD133 were confirmed by Real Time-PCR.

Conclusion: We showed that CD133 antigen can be used as a suitable marker for CSCs detection in breast carcinomas. The analysis was carried out by different methods: flow cytometry, immunohistochemistry and Real Time PCR quantification. Our data were confirmed by all three methods, and the results are overlapping.

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## CIRCULATING TUMOUR CELLS: TECHNICAL AND CLINICAL CHALLENGES

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Circulating tumour cells (CTCs) may be detected in the blood of patients with epithelial tumours using different analytical approaches. The relative number of CTCs is low and they include a heterogeneous population of cells with diverse biological and molecular characteristics, often different from those of the respective primary tumour. Until recently, they have been difficult to detect and, even though discordant results have been reported when different methods of detection were used, they may provide prognostic and predictive information.

Several antibody-or molecular-based CTC detection methods have been developed, offering hope for individualized risk assessment by utilizing CTCs as biomarkers of disease progression and drug response. Pilot studies have also shown that utilizing methods that permit, besides enumeration, a molecular characterization of CTCs, one could better identify high-risk patients, predict response to targeted therapies, analyze gene expression profiles (in order to identify new potential drug targets), and increase our knowledge of the metastatic process.

In order to achieving these goals, there is a great need for development of more sensitive technologies able to avoid stress on the cells during manipulation, in order to preserve cell viability and proliferation capability and to facilitate subsequent in vitro culture for further molecular analysis of CTCs. Of course, the cost of such methods is another important issue that should be addressed in view of larger clinical applications.

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## TRABECTEDIN: A SOMETHING NEW IN ONCOLOGY

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Trabectedin is a tetrahydroisoquinoline alkaloid that was initially isolated from the marine ascidian *Ecteinascidia turbinata* and is currently prepared synthetically. Trabectedin's mechanism of action seems to be different from that of the available DNA-damaging agents used in cancer chemotherapy to date. In contrast to traditional alkylating agents that bind guanine at the N7 or O6 position in the DNA major groove, trabectedin binds to the exocyclic N2 amino group of guanines in the DNA minor groove. Several

studies have been conducted on cell lines with well-defined defects of DNA repair mechanisms suggesting a role for transcription-coupled nucleotide excision repair (TC-NER) and homologous recombination in the cytotoxic activity of trabectedin. In contrast to traditional alkylating agents trabectedin in NER+ cells induced a $G_2M$ block of the cell cycle, while in NER−cells did not. Trabectedin inhibits the *in vitro* production of the proinflammatory mediators CCL2 and interleukin (IL)-6 by monocytes, macrophages, and tumor-associated macrophages isolated from ovarian cancer biopsies. The modulation of cytokines and chemokines occurs at the transcriptional level, thus indicating that the mechanism of trabectedin on transcription regulation can be effective both in cancer cells and in some normal cells, which produce factors that are relevant for the tumor growth and progression. Trabectedin has been shown to be a clinically valid option for the treatment of advanced soft tissue sarcomas or relapsed platinum-sensitive ovarian cancer.

## HUMAN TH1 AND TH17 LYMPHOCYTES IN CHRONIC INFLAMMATORY DISEASES

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Classically, naive CD4+ T cells have been divided into two main lineages, Th1 and Th2 cells. Th1 cells produce IFN-, the cytokine IL-12 being critical for their differentiation. By contrast, Th2 cells produce IL-4, IL-5 and IL-13 and their development is started by IL-4 signalling. More recently, a third subset of CD4+ T cells which produce IL-17 has been described, which was named as Th17. We showed that, in addition to cells producing IL-17 alone (Th17), there is a number of T cells that co-produce IL-17 and IFN- (Th17/Th1). We also found that these two cell types express both RORC and T-bet and that Th17 cells could be shifted to Th1 by the addition of IL-12 suggesting their flexibility. Moreover, we found that CD161 is a marker of human Th17, in comparison with Th1 or Th2, clones. Accordingly, all IL-17-producing cells were found to be included within the CD161+ fraction of adult circulating CD4+ T cells. When CD161+ or CD161-cells were sorted from UCB naïve CD4+ T cells and activated in presence of IL-1beta plus IL-23, IL-17-producing cells could be developed only from the CD161+ fraction. In addition, we found that not only CD4+TCRβ+, but also CD8+TCRβ+, CD4-CD8-TCRβ+, and CD4-CD8-TCR+ circulating cells that produce IL-17 express the distinctive marker CD161 on their surface. Accordingly, we also demonstrated that CD161 expression identifies also CD8+ and CD4-CD8-UCB T cells that already express RORC and IL-23R and that can be differentiated into Th17 cells in the presence of IL-1 and IL-23. Taken together these data allow to conclude that T-cell subsets able to produce IL-17, as well as precursors of IL-17-producing T cells, exhibit surface expression of CD161.

## IMAGING CYTOMETRY REQUIRES THE THIRD DIMENSION AND HIGH SPATIAL AND TEMPORAL RESOLUTION

**Diaspro A.,[1,2,3] Bianchini P.,[1] Cella Zanacchi F.,[1] Harke B.,[1] Vicidomini G.,[1] Galiani S.,[1,2] Lavagnino Z.,[1,2] Chacko J.,[1,2] Ronzitti E.,[1] Faretta M.,[3] Furia L.[3] and Mondal PP.[4]**

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Two-photon excitation (2PE) fluorescence imaging cytometry is a powerful far-field optical approach for the study of the three-dimensional (3D) and dynamic properties of biological systems. The main advantages are given by better penetration in scattering samples and low overall phototoxicity/bleaching coupled to intrinsic 3D optical sectioning properties, while the main drawback lies in the loss of resolution and signal efficiency with respect to the 1PE case. For such reasons we decided to couple 2PE with STED microscopy (STimulated Emission Depletion) and SPIM (Selective Plane Illumination Microscopy). In the former case we aim to augment resolution and at the same time to improve the sample penetration capability of the STED approach. In the latter we aim to boost SPIM penetration depth in thick scattering samples. Results and related characterizations have been obtained by means of 2PE STED-CW and 2PE SPIM adapted architectures available at the Italian Institute of Technology. Other extensions of 2PE are in progress within the farfiled optical super resolution framework. This work was supported by grants from IIT, IFOM-IEO, MIUR PRIN 2008JZ4MLB, European Project SMD FP7-NMP 2800-SMALL-2 proposal no. CP-FP 229375-2.

## MICRORNAs AS NEW THERAPEUTIC TARGETS AND TOOLS IN CANCER

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MicroRNAs (miRNAs) are a class of endogenous, noncoding small RNAs that negatively regulate gene expression at the post-transcriptional level. Several studies have provided evidence that abnormal expression of selected miRNAs is associated with the pathogenesis of cancer. As they can act as either oncogenes or tumor suppressors, miRNAs have been proposed as potential new therapeutic targets or tools for cancer therapy. A significant body of the experimental data collected to date indicate that specific miRNA inhibition or replacement can successfully modify the proliferative and invasive properties of tumor cells. In addition, a direct involvement of miRNAs in drug resistance has been reported, underlying an entirely new mechanism by which tumor cells may be refractory to the treatment with cytotoxic agents. Based on these findings, in the therapeutic setting, interference with cancer-

specific miRNAs could be exploited not only to produce a direct anticancer effect but also to improve the response of tumor cells to conventional treatments. Overall, manipulation of miRNA functions, either by mimicking or inhibiting them, is emerging as a highly promising therapeutic strategy. However, before miRNA-based therapeutics enters the clinical armamentarium, important issues concerning specific delivery to cells/tissues of interest, safety as well as pharmacokinetic profiles needs to be addressed.

## CELL CYCLE AND APOPTOSIS

### THE EFFECT OF LOW LEVEL LASER IRRADIATION ON HUMAN OSTEOBLASTLIKE CELLS GROWTH: AN IN VITRO ANALYSIS

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Low-level laser irradiation (LLLI) has been shown to be effective in the promoting the proliferation of different cells. The aim of this study was to examine the *in vitro* effect of a single or a multiple doses of LLLI on proliferation of the human osteosarcoma cell line, SAOS-2. The cells were divided in groups and then exposed to different doses of LLLI with a semiconductor diode laser (659 nm; 9.6 mW power output; 1, 3 J/cm$^2$) for 1 day and 3 consecutive days, respectively. Cellular proliferation was assessed at different time up to 7 days of culturing. Moreover, Fluorescein diacetate (FDA) assay and cytoskeleton-immunofluorescence were performed at the end of experiment. The obtained results showed an increase in proliferative capacity of SAOS-2 in once-irradiated cells, as compared with not irradiated cells. In particular, a significantly higher cell growth in the group irradiated with 3 J/cm$^2$ for three consecutive days was detected if compared with the other irradiated-groups or dark control. FDA assay showed no differences of cell viability between all experimental cases and dark control and the detection of α-tubulin revealed that cells original morphology was not changed by laser irradiation. This preliminary study suggests that: LLLI affected positively SAOS-2 proliferation; cells were not apparently damaged by laser application; and repeated irradiations were necessary to observe a markedly enhancement of SAOS-2 growth. Further research will be required to validate these data. Our aim will be to determine the potential of LLLI as a new approach for promoting bone regeneration onto biomaterials.

### APOPTOSIS AND CELLULAR STRESS IN PERIPHERAL LYMPHOCYTES MAY BE USED AS EARLY BIOMARKERS OF MULTIPLE SCLEROSIS DISEASES

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Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system, the aetiology of which, although not completely known, involves changes in the inflammatory process and in the autoimmune system. The aim of the present study was to identify molecular markers of MS in lymphocytes of 19 MS patients and 13 healthy individuals. We focus on markers of apoptosis, cellular stress and DNA damage. Apoptosis was evaluated by flow cytometry looking on two typical hallmarks, that are phosphatidylserine exposure (revealed by Annexin V-FITC) and membrane permeability to Propidium Iodide (PI). The analysis is able to discriminate between viable and dead cells; moreover, cells in early and advanced apoptosis are clearly distinguishable. As for stress parameters, we have considered the synthesis of poly(ADP-ribose) (PAR) that is produced under stress conditions by the enzyme poly(ADP-ribose) polymerase-1 (PARP-1), and the phosphorylation of the histone H2AX. Both PAR and phosphorylated H2AX (gH2AX) are detected by immunofluorescence. Our results demonstrated that the number of apoptotic cells in patients is significantly higher than in healthy individuals, thus suggesting that apoptosis could affect MS lymphocyte function. Of note, we detected for the first time a net increased level of PAR and gH2AX in lymphocytes of patients compared to healthy subjects. Patients were subdivided in three groups, according to the neuroimaging (MRI)-based classification of disease phase. Remarkably, we found a positive correlation between the level of gH2AX and MS aggressiveness and a correlation between phosphorylation of H2AX histone and disease activity. Further experiments are in progress to include a large number of patients and to support these cell-based data with molecular investigation on parallel (already stored frozen) aliquots of patients/controls samples.

### INCREASED SOD1 NUCLEAR LOCALIZATION IS ASSOCIATED WITH HIGHER LEVELS OF REACTIVE OXYGEN SPECIES AND EARLY APOPTOSIS IN LYMPHOCYTES OF SPORADIC ALS PATIENTS

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The mechanisms involved in motor neuron degeneration in Amyotrophic Lateral Sclerosis (ALS) remain mainly unknown. Current evidence indicates that wild-type SOD1, under conditions of cellular stress, is implicated in a significant fraction of sporadic patients (SALS), which represents 90% of total ALS patients. Although motor neurons are the specific target, increasing observations suggest that some dysfunctions are also present in lymphocytes. In previous experiments we described an abnormally high level of SOD1 transcript in lymphocytes of SALS patients despite a decreased SOD1 protein expression in the total lysate.

The aims of this work were: 1) to understand whether the discrepancy between SOD1 mRNA and protein expression is due to a different cellular distribution of the protein; 2) to investigate whether the different SOD1 distribution is associated with increased oxidative stress and cell damage. Nuclear and cytosolic fractions from 19 SALS and 17 healthy controls (HC) were obtained and SOD1 expression evaluated by western blotting (WB). SOD1 localization was detected by immunofluorescence. Total SOD1 expression, reactive oxygen species (ROS) and early apoptosis were evaluated by flow cytometry.

WB experiments and immunofluorescence showed a significant increase of SOD1 protein nuclear localization in SALS patients. Total SOD1 expression was significantly higher in SALS cells compared to HC. A significant increase in oxidative stress level and percentage of cells in early apoptosis were found in patient lymphocytes.

In this work we found an increased nuclear SOD1 expression in SALS patients that was associated with a higher level of oxidative stress and rate of apoptosis. This observation reveals new scenarios on SOD1 nuclear function and its implication in ALS pathogenesis.

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MORPHOLOGICAL AND FUNCTIONAL STUDIES ON HETEROGENEOUS CELL POPULATION DERIVED FROM HUMAN OVARIAN FOLLICULAR LIQUID

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The origin of oocytes and primary follicles in ovaries of adult mammalian females is still a matter of dispute. The components of new primary follicles, primitive granulose and germ cells, differentiate sequentially and de novo from mesenchymal progenitor cell residing in the ovarian tunica albuginea (TA). It appears that mesenchymal progenitor cells contribute to the generation of epithelial cells similar to granulosa cells (GCs). The aim of this work is to study the morphological and functional characteristics of cell populations collected from human ovaries and to analyse particularly the growth of mesenchymal-like GCs *in vitro* in medium without any growth factor (such as leukemia-inhibiting factor) beyond 20 days. The interest in this study is to use waste biological liquid picked-up during human assisted reproduction techniques as a new possible source of mesenchymal stem cells (MSCs) to use in *in vitro* oocytes maturation techniques. Cells obtained from human follicular fluid grow *in vitro* in minimal medium condition, without any kind of growth factor. Morphological analysis revealed an heterogeneous cell population, with cells displaying a fibroblast-like, epithelial-like and also neuron-like shapes. The characteristics of fibroblast-like cells were similar to mesenchymal stem cells, while keeping also some aspects of ovary granulosa cells. Using immunocytochemistry and flow cytometry these cells have been shown to be positive for several mesenchymal stemness markers, including CD90, CD73, CD44, CD105, but not cytokeratins and they are also negative to CD34, CD45 and other aspecific markers. To isolate CD44+ cells of ovary-derived stem cell population, in this study we have performed experiments using immunomagnetic procedure. Parallel experiments were done on MSC cells from human bone marrow that served as positive control. Cell proliferation activity at different times in minimal culture conditions and colony forming unit capacity were evaluated too. After 15–20 days in culture, BrdU incorporation show about 20% proliferating cells less then after 1 week. Colony formation was observed after 13 days in culture. The multipotency of the subset of CD44+ cells was also established by *in vitro* differentiation into other cell types, such as osteoblasts. We have demonstrated the possibility that cells with mesenchymal plasticity can be isolated simply collecting waste follicular fluid, avoiding scraping of human ovaries. We also demonstrated the presence of a small subpopulation of neural-like cells in culture derived from the waste ovarian follicular fluid.

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CELL DEATH MODALITIES INDUCED BY SINGLE OR COMBINED TREATMENTS WITH RADIATION AND TEMOZOLOMIDE IN GLIOBLASTOMA CELL LINES

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Malignant gliomas are the most frequently occurring primary tumours of the central nervous system and glioblastoma multiforme constitutes one of the most aggressive and lethal malignancies. Despite the recommended treatment currently used in clinical, its survival benefit remains unsatisfactory and it continues to present an enormous therapeutic challenge. In this study single or combined treatments of radiation and temozolomide (TMZ) were performed to understand which multimodality treatments could afford an improvement in the efficacy of therapy in terms of tumour cell death. The aim of this study was the evaluation of cell death modalities induced by radiation and TMZ in different glioblastoma cell lines. Human glioblastoma U251 and U87 cells were irradiated at doses from 2 Gy to 20 Gy with γ-rays using a Cobalt-60 source (Struttura Complessa di

Radioterapia Oncologica, IRCCS Policlinico S.Matteo, Pavia). Sham irradiated cells (0 Gy) were performed as control. For combined applications, before irradiation cells were treated with TMZ, the chemotherapeutic drug used in clinical for the treatment of glioblastoma. Flow cytometry was used to detect death of tumour cells induced by irradiation and combined treatment after 24 and 48 hours. During cell death, characteristic changes of the morphology can be detected by (FSc) forward scatter/(SSc) side scatter properties of the cells. To distinguish apoptotic from necrotic cells the exposure of phosphatidylserine by apoptotic and necrotic cells was analyzed by binding of FITC-labeled AnnexinV, and necrosis was differed from apoptosis by co-staining with propidium iodide (PI). A third population was identified and classified as mild damaged cells that showed an intensity of PI staining lower than necrotic cells. Results showed that apoptosis is the prominent cell death forms in both cell lines. A dose-dependent increase in apoptotic cells was observed, in particular after 48 hours from irradiation. Combined treatments seemed to be more effective especially on necrosis than single treatments.

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## HMGB1 AND HSP70 INVOLVED IN RADIATION RESPONSE IN HUMAN GLIOBLASTOMA MULTIFORME CELLS

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Immunotherapy or the induction of immunogenic glioblastoma cells by standard therapies could be a promising strategy to prevent tumour recurrence because of the high degree of selectivity and the long-lasting memory of the immune system. Malignant cancer cells respond to RT and/or CT by distinct forms of cell death that may lead to a specific activation of the immune system due to the exposure of immunogenic factors on the cell surface or the release of immunogenic signals into the extracellular space. These factors can elicit the immune activation by fostering the uptake of the tumour cells by dendritic cells (DC) and their consecutive contact with T cells may lead to specific and, most importantly, long-lasting anti-tumour immunity. In fact, this anti-tumour immunity should kill cancer stem cells, resistant to the RT, and (micro)-metastases and keep residual tumour cells in check. The identity, the presence and the amount of these immunogenic signals as well as the tumour cells' immunogenicity determining molecules in glioblastoma are still not known.

The aim of this work has been to study in glioblastoma cell lines immunogenic danger signals involved in the activation of immune cells, such as high mobility group box-1 (HMGB1) protein, heat-shock protein 70 (Hsp70), and calreticulin. We observed the presence, release and exposure on the surface of human glioblastoma T98G cells of HMGB1 and Hsp70 after different doses of gamma radiation and therapeutically relevant concentrations of temozolomide by Western blotting and immuno-cytochemistry. The data suggest a modulation of the expression of these danger molecules after standard treatment of glioblastoma cells, suggesting that those signals play crucial roles in radiation or chemotherapeutic responses leading to specific and long lasting anti-glioma immune responses.

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## SET UP OF A MULTIPLE DETECTION SYSTEM FOR ANALYSIS OF CELL APOPTOSIS

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The p53 tumor suppressor has a central and fundamental role in maintaining cell integrity and preventing malignancies. Due to this crucial position, p53 is finely regulated and the main players in this activity are MDM2 and MDM4 proteins. MDM2, an E3 ubiquitin ligase, ubiquitinates p53 and targets it to the proteasome for degradation. Moreover, it represses p53 transcription activity. MDM4 represses as well p53-mediated transcription but, it is not capable of ubiquitin ligase activity. Under normal growth conditions, MDM4 enhances the E3 ligase activity of MDM2 towards p53 by heterodimerizing with MDM2. On the other hand, upon stress conditions MDM4 can favor p53 mediated apoptosis at the mitochondria by bridging its binding to the antiapoptotic Bcl-2. To validate these MDM4 and MDM2 activities, we analyzed cell apoptosis using mutant and wt proteins. We first set up the correct experimental conditions to test the apoptotic response in genetically modified cells. We used an apoptotic assay based on the binding of AnnexinV to externalized phosphatidylserine (PS), a hallmark of early apoptosis. Annexin V is commonly conjugated to fluorescein (a.k.a. FITC), so apoptosis detection in green fluorescent protein (GFP)-expressing cells can be problematic. Indeed, the spectral overlap between the emission profiles of these two fluorophores makes it impossible to differentiate between signals thus compromising data quality. We therefore set up a flow cytometry detection analysis using Annexin V bound to cyanine 3, GFP signal in transfected cells and 7-AAD measurement as third parameter. Using this test, we could evaluate the percentage of apoptosis among transfected cells linking the induction or suppression of apoptosis specifically to the action of our transfected proteins.

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## BONE CALCIFIED MATRIX ONTO ZINCCONTAINING BIOACTIVE GLASSES

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The concept of bioactive glass (bioglass) was developed and tested for the first time by Hench at the beginning of

the '70s. The common characteristic of bioglass is that, upon implantation, their surface undergoes complex kinetic modifications leading to the formation of an amorphous calcium phosphate layer which crystallizes into a biologically active hydroxycarbonate apatite (HCA) phase, chemically and structurally equivalent to the mineral phase in bone. The bioactivity of glass can also be improved by adding intermediate or modifying oxides to the base compositions. It was shown that the addition of ZnO to standard bioglass could stimulate osteoblast proliferation and differentiation, thus improving the implant's ability to bond with bone. The aim of the present study was to perform a detailed investigation of bioglasses containing 0.4 %wt ZnO, in terms of osteoblast morphology, proliferation, and deposition of a mineralized extracellular matrix. Pure silica, 58S and 58S with 0.4%wt ZnO disks were synthesized by sol-gel routes. The human osteosarcoma cell line SAOS-2 was seeded on disks. Scanning Electron Microscopy (SEM) observations showed that cells more homogeneously covered the surface of the 58S-Zn0.4 sample than that of the silica and 58S disks. In comparison with the silica and 58S disks, the deposition of bone proteins throughout the 58S-Zn0.4 samples was considerably enhanced ($p < 0.05$). The mineralization of the extracellular matrix produced by SAOS-2 cells was considerably greater (almost 2-fold greater) on 58S-Zn0.4 than on silica and 58S disks ($p < 0.05$). This study confirms that a 0.4%wt zinc oxide in 58S samples improves the environment and favours osteoblast proliferation and function.

INCREASE IN p21CDKN1A PROTEIN LEVELS BY PROTESOME INHIBITORS DOES NOT AFFECT THE APOPTOTIC RESPONSE AFTER DNA DAMAGE

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p21CDKN1A is a well known cell cycle inhibitor, which plays an important role also in other pathways, including transcription regulation, apoptosis, and DNA repair. Several pharmacological approaches have been developed to contrast tumor cell proliferation, by exploiting the p21 ability to induce cell cycle arrest. In fact, inhibitors of histone deacetylases (HDAC), and inhibitors of proteasomal machinery have been shown to increase p21 protein levels with the consequence of impairing tumor cell growth. However, this type of intervention has been regarded as potentially dangerous, when applied in concomitance with typical anticancer drugs inducing DNA damage. In fact, high p21 levels have been shown to inhibit the apoptotic program and to allow the repair of DNA damage, thereby rendering tumor cells less susceptible to the killing activity of the latter drugs. Thus, p21 protein levels may greatly influence the outcome of chemotherapy. We have previously found that HDAC inhibitors did not significantly affect initiation of nucleotide excision repair (NER) in the presence of p21 protein. In contrast, no clear information is available on the effect of proteasome inhibitors on DNA repair. Here, we have investigated whether the presence of elevated p21 protein levels induced by the proteasome inhibitor MG132, may affect the NER and the apoptotic process. We have analyzed the recruitment of NER proteins and p21 to localized DNA damage sites, and determined NER efficiency in normal, as well as in p21-null human fibroblasts. The results have shown that MG132 induced the persistence of XPC, PCNA and p21 protein, at local DNA damage sites. The persistence of p21 at DNA damage sites did not appear to significantly affect the recruitment of other PCNA-interacting NER factors, like DNA polymerase delta. However, NER efficiency was reduced and cell death was increased by the proteasome inhibitor. These results suggest that MG132 affects NER efficiency and induce apototic cell death, independently of the presence of p21 protein. [Work supported by AIRC].

## ENVIRONMENTAL SCIENCES AND TOXICOLOGY

ABIOTIC AND BIOTIC FORMATION OF RED ELEMENTAL SELENIUM NANOPARTICLES: POTENTIALITIES OF FLOW CYTOMETRY

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In natural environments, the toxic $SeO_4^{2-}$ and $SeO_3^{2-}$ ions undergo abiotic and biotic reduction with the production of elemental red Se0 nanoparticles. Multiple detoxification processes may be catalyzed during selenite and selenate reduction by aquatic microorganisms. Assessing the effects of Se-nanoparticles released in the environment requires continuous methodological improvements for a better understanding of their persistency and eco-toxicity. In this study, we combined the Hydride Generation-Atomic Absorption Spectroscopy and Flow Cytometry to evaluate the abiotic and biotic kinetics of formation and the size distribution of Se-nanoparticles. Under abiotic conditions, the main factors affecting the growth of $Se^0$ nanoparticles were the amount of available reducing agent, the initial amount of SeIV and the solution pH. Under biotic conditions, the kinetic of formation was quite slow (4 weeks) and drastically enhanced by the increase of bacterial cell number which followed the addition of glucose as growth substrate. Overall, the nucleation and morphological evolution of Se-nanoparticles were clearly detected by Flow Cytometry according to the side and forward scatter signals at small and large angles. The observed particle size ranged from about 100 nm, corresponding to the instrumental lower detection limit, up to

over 1 μm, commonly considered as the upper size limit of the colloidal matter in natural waters. Being Flow Cytometry a convenient method for a rapid and multiparametric particle size evaluation, we found that the adsorption on cell walls and the incorporation into bacterial cells, considered as common detoxifying microbial responses, could prevent the aggregation and formation of larger Se-nanoparticles.

## FLOW CYTOMETRY AS A RAPID AND MULTIPARAMETRIC TOOL TO SUPPORT THE HYDROGEOCHEMICAL ASSESSMENT OF GROUNDWATER STATUS

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The protection and regulation of groundwater resources rely on a hydrogeochemical characterization to understand the impact of diffuse pollution from intensive agriculture, waste disposal and point source pollution from urban and industrial sources. In this context, methodological advances in geomicrobiology are also of growing importance to complement water quality issues. The objective of the study was to identify which of the microbiological parameters could serve as valuable indicator of groundwater quality. We performed a field-scale analysis along the southern Sabatini Mts. aquifer (50 sampling sites) in which Pleistocene volcanic products overlay gravel and silty clay layers. The main physicochemical parameters were determined (redox status, pH, conductivity, T, DO, alkalinity, major and trace elements, including arsenic). Furthermore, all samples were analysed by Flow Cytometry for the evaluation of the total abundance and live/dead cell ratio of the free-living microbial community. The hydrogeochemical characterization defined an alkaline-earth bicarbonate type. The variability of bacterial abundance was nonetheless high, ranging from $10^4$ up to $10^6$ cells/ml. A direct correlation of the total cells with the main parameters of water quality was not found. However, the percentage of dead bacterial cells showed a significant correlation with arsenic concentration. In conclusion, microbiological data may represent a valid complement to address questions of origin and attenuation of pollution. Moreover, Flow Cytometry seems an appropriate analytical tool to provide rapid and multi-parametric data for a better understanding of the biogeochemical processes at the microscale level.

## UTILITY OF THE BAT TEST IN THE CLINICAL ANALYSIS OF AQUATIC ALLERGENS

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The increased consumption of raw fish in the European diet has contributed to the development of human Anisakis allergy. Anisakis simplex is a nematode that parasitizes marine mammals at the larval stage 3 (L3) and is found in the celomic cavity and viscera of adult sea fish and cephalopods and from those organs, the larvae often can reach the host muscles, that are consumed by humans. Humans are an accidental host. Anisakiasis occurs mainly when the parasitized fish are consumed raw, undercooked or marinated. Anisakis plays a role in several gastric and intestinal diseases. Following ingestion, the larvae can penetrate the human gastrointestinal mucosa, causing ulcerations, eosinophilic granulomas and digestive symptoms: abdominal pain, vomiting and nausea.

Aim: To evaluate the sensitization to Anisakis simplex using Flow-cytometry analysis of *in vitro* BAT (Basophil Activation Test).

Methods: In Patients presenting to the Allergology Division with abdominal pain, we evaluated basophil degranulation. We evaluated the percentage of expression of CD63, CD123, HLADR by cytometric analysis by skin prick test with small diameter spot. We found the BAT test was positive to anisakis in 75% patients, to confirming prick test data. Possibly, the negative test patients, could have shown a cross reactivity with dust mites.

Conclusion: We consider that BAT is a reliable new additional in vitro method test for Anisakiasis.

## POLYUNSATURATED ALDEHYDES EFFECT ON MICROBIAL COMMUNITY COMPOSITION AND DYNAMICS IN THE NORTHERN ADRIATIC SEA

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Polyunsaturated aldehydes (PUAs), produced by diatoms as secondary metabolites, are known to act as infochemicals and allelochemicals shaping plankton communities and determining the fate and transfers along the pelagic food web. During a cruise in February 2008 in the northern Adriatic Sea we tested the effect of PUAs, octadienal and a mix of octadienal and heptadienal, on natural bacterial communities using flow cytometry and MAR-CARD-FISH. In order to contextualize the experiment, picoplankton (autotrophic and heterotrophic) distribution and dynamics were also investigated using flow cytometry at 32 stations.

Within the autotrophic picoplankton *Synechococcus* represented the most abundant group (4.29 x 104 cell $ml^{-1}$) in the northern and oligotrophic stations along Croatia. Picoeukaryotes (2.54 × 103 cell $ml^{-1}$) and heterotrophic bacteria (7.85 × 105 cell $ml^{-1}$) were mainly concentrated at surface and at Italian coastal stations. PUAs incubation showed little or no effect on total bacterial concentration. The most abundant groups were SAR11 and CFB (22% of total cells). The proportion of Alfaproteobacteria and the CFB showed dramatic changes after PUAs incubation (70% decrease in the Octa and 45 and 80% in the MIX treatment

after 24 and 48h of incubation, Alfa and CFB respectively). Metabolic activity indicated a group-specific reaction to the PUAs, with Alfaproteobacteria the most affected group. Metabolic activity of all bacteria groups was negatively affected after 24h of MIX exposure while after 48h Roseobacter metabolic activity increased. Hence, PUAs appear to have a role in determining bacterial community composition and metabolic activity directly by favouring some bacterial groups, or indirectly, by slowing down some others.

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CYTOLETHAL DISTENDING TOXIN FROM TWO DIFFERENT STRAINS OF *CAMPYLOBACTER JEJUNI* INDUCES DISTINT DEATH PATHWAYS IN HeLA CELLS.

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*C. jejuni* is the most common cause of bacterial gastroenteritis in humans. Several virulence factors such as the ability to adhere and/or invade human enterocytes and synthesis of toxins, in particular way the cytolethal distending toxin (CDT), appear essential in the infection process. The aim of our research is to evaluate the sequence of lethal events in HeLa cells exposed to the G2 blocking CDT from *C. jejuni* ATCC 33291 and *C. jejuni* ISS3 and characterize these processes as necrosis, apoptosis or both. Sonicated and centrifuged filtrates were diluted, added to HeLa cell monolayers and examined at 24 h intervals for 96 h.

HeLa cells were analysed for different aspects. At 72 h we detected mitochondrial impairment both for mitochondrial membrane potential (by TMRE) and for degree of cardiolipin peroxidation (by NAO). Human cervical carcinoma HeLa cells possess wild-type p53, although p53 protein was almost undetectable in untreated control cells. Of note, after CDT treatment of HeLa cells, an increase of the p53 level was observed, particularly for *C. jejuni* ATCC 33291 strain and after 48 h. Furthermore bcl-2 analyses highlight a reduction of 23% and 43% in intracellular protein levels for *C. jejuni* ATCC 33291 and *C. jejuni* ISS3 strains, respectively. Altogether the data characterize CDT-induced cell death principally as apoptosis p53-dependant. Recently, it was suggested that p53 might be involved in regulation of CD59 expression in inflammation thus protecting host cells from innate complement lysis: our data show a downregulation of CD59 of about 35% in treated HeLa cells This may be a defensive mechanism that allows infected host cells to be targeted by complement.

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DETECTION OF ORAL PATHOGEN MICROORGANISMS BY FLOW CYTOMETRY

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The oral cavity of healthy individuals contains many types of different bacterial species. They can associate to form biofilms, becoming resistant to mechanical stress or antibiotic treatment. These bacteria can be pathogenic in responses to changes in the environment or other triggers in the oral cavity. Microbial composition of multispecies communities in oral biofilm was mostly studied by molecular techniques and confocal laser scanning microscopy (CLSM). A new approach may be the use of flow cytometry (FCM), a versatile powerful tool for investigating characteristic features of individual cells in a heterogeneous population with fluorochrome-linked polyclonal and monoclonal antibodies. To this purpose in this work, polyclonal antibodies were developed in rabbits against *Streptococcus* mutans ATCC25175, *Streptococcus* oralis ATCC9811, *Porphyromonas* gingivalis ATCC33277, and *Fusobacterium* nucleatum ATCC25586, species implicated in oral infection diseases. The sensitivity of our antibodies tested by FCM showed a specific fluorescence intensity of each antibody against each species, as also confirmed by CLSM observations. The specificity of each antibodies against other species was evaluated both by ELISA and FCM.

In conclusion, the application of FCM in combination with specific polyclonal antibodies could be a helpful method for the identification of bacteria in oral biofilm.

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REAL TIME PCR: A BIOMOLECULAR METHOD TO INVESTIGATE GENE EXPRESSION AND INTRASPECIFIC BIODIVERSITY

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Real-Time (RT) PCR is a bio-molecular method to measure gene expression in cellular systems; it's an up-grade of traditional PCR, based on fluorescent detection. Recently, RT-PCR has also been applied in new areas of interest such as: viral quantification and gene expression, array verification, drug therapy efficacy, DNA damage measurement, quality control and assay validation, identification of DNA contaminants in food matrix, pathogen detection and genotyping. This technique is largely used in our laboratory; in our last work (Matteucci M et al., 2011) it has been used to detect the expression of fatty acid desaturase (*FADs*) genes since it is useful to investigate organisms with high intraspecific biodiversity like *Olea europaea L.* In present research RT-PCR has been used to investigate the presence of *FADs* in different tissues of olive tree and their different expression during the fruit development, the oleogenic period and the cold stress. The unsaturated fatty acids produced, indeed, contribute to quality of oil and to cold tolerance of tree. The relative abundance of transcripts and their pattern expression have been observed to be different in genotypes with different in cold sensitivity. In this work, gene expression data are matched with cyto-histological analysis and NMR spettroscopy that allowed us to better understand the physiological problem of cold stress. Histological analysis showed, for the first time, the involvement of chloroplast in oil production in olive drupes. The comparison between molecular and metabolomics data, indeed, allowed

us to study the "cold-enzymes" (in chloroplast and in ER) from transcripts expression up to metabolite production. Therefore, it is recomanded that this technique should be compared with other research methods, such as flow-cytometry in the future studies in this field.

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BORON NEUTRON CAPTURE THERAPY (BNCT): AN '' *IN VITRO*'' CHARACTERIZATION STUDY OF THE REFRACTORY CELL FRACTION

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Extracorporeal liver BNCT improves life expectancy in diffuse metastases from coloncarcinoma. Nevertheless, long term surviving is compromised by the late appearance of recurrences, derived from a small fraction of refractory cells spared by the neutron irradiation treatment. The existence of this cell fraction was also confirmed by our *in vitro* studies. Such ineffectiveness is assumed to be caused by inadequate absorbed radiation dose due to the insufficient intracellular $^{10}B$ content.

To test this hypothesis, the relationship between cell proliferation and boron uptake capability is investigated *in vitro* in order to characterize this resistant cell fraction and to optimize the efficacy of the therapeutic procedure.

Experiments are performed on the DHDK12TRb (DHD) coloncarcinoma cell line simultaneously cultured with boronophenylalanine ($^{10}BPA$) and bromodeoxyuridine (BrdU), that allows proliferating cells identification by means of its immunodetection. The plating efficiency test performed on cells after neutron irradiation exposure identify the proliferative status of the surviving cell fraction.

Our result suggest that there is a correlation between cell proliferation and BPA uptake. Cells, proliferating at the time of BPA exposure, are highly sensitive to BNCT. Conversely only non proliferating cells can survive BNCT treatment.

This information may find utility in the design of BNCT treatment plan in the clinical procedure.

In order to overcome the problem of recurrences studies must be addressed to characterize this cell subpopulation and to develop boronated compounds that can be captured by cells independently from their proliferation status.

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REACTIVE NITROGEN AND OXYGEN SPECIES (RNS/ROS) PRODUCTION IN RESPONSE TO POLYUNSATURATED ALDEHYDES IN THE MARINE DIATOM *SKELETONEMA MARINOI*

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Some marine diatoms produce PolyUnsaturated Aldehydes (PUAs) as secondary metabolites through a lipoxygenase-mediated pathway. The teratogenic effects of these compounds on several marine organisms have been long documented. PUAs can induce several toxic effects at high concentrations in diatoms themselves (e.g. growth inhibition), and at sublethal concentrations they can function as stress signals triggering sensitization and increased resistance to toxicity through the involvement of nitric oxide (NO) and calcium. PUAs may also act as 'infochemicals' in modulating interactions between diatoms and surrounding organisms during phytoplankton blooms at sea. In addition to this, they are supposed to be used by diatoms themselves as intra-populations signals leading to increased usage of the limiting resources at the end of blooms. When conditions become extreme, they also trigger massive cell death through a mechanism closely resembling apoptosis.

Here we report the response of the marine diatom *Skeletonema* marino*i* upon exposure to different PUAs in terms of Nitric Oxide (NO) and Reactive Oxygen Species (ROS) production. Contrarily from previous results obtained with a non PUA-producing and non bloom-forming species, we observed a general decrease in NO production in treated samples proportional to PUAs concentrations. Instead, ROS production was induced. Light stress, instead, was observed to induce NO production. These results might suggest a species-specific response to PUAs, related to the ability of a certain species to produce and consequently encounter these compounds in the natural environment. Moreover, a stress-specific type of response in NO production seems to be present in *S. m*., and this suggests different cell signal pathways in response to adverse environmental conditions.

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REFINING THE METHODOLOGY FOR COMPLEX GENOME DISSECTIONS BY CHROMOSOME ARMS FLOW SORTING

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Complex genomes crop such as bread wheat are of main importance to feed billions human beings all over the world. This important commodity is an hexaploid species (*Triticum aestivum* L. $2n = 6x = 42$ AABBDD) with a huge genome of 17 Gbp about, most of which is made of repetitive DNA sequences (more then 80%). Three homeologus genomes and large interdispersed repetitive sequences make genomics a difficult target to pursue. A flow sorting approach to separate specific chromosomes and chromosome arms in suspension is the only chance to dissect complex genomes. Chromosome sorting is a remarkable source of DNA that can be used for physical gene mapping, isolation of molecular markers, and construction of chromosome-specific DNA libraries.

Here we describe the method to obtain 5AS chromosome arm specific DNA by flow cytometry analysis, flow sorting, proteinase K digestion and GenomiPHI DNA amplifi-

cation All the proci sederu have been optimized in terms of analysis accuracy, time and yield such as: (1) accumulation of cells in metaphase, (2) preparation of chromosome suspensions, (3) flow analysis and sorting, (4) purity control of sorted chromosomes and (5) processing of their DNA. In particular, we have: improved the precision of DAPI chromosome staining; applied a simple and fast FISH method for purity estimation of sorted fractions; adopted a faster processing method to purify and obtain chromosomes specific DNA of high molecular weight. These new achivements will contribute to make easier and more effective the chromosome approach for wheat and other crop.

This work is supported by MIPAAF, Project MAPPA 5A (D.M. 7398/7303/08)

## FLOW CYTOMETRIC ANALYSIS OF POLYPLOIDY INDUCTION IN ORCHID IN VITRO CULTURES

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Orchids are part of the monocotyledonous *Orchidaceae* families, which is one of the most improved families listing more than 150.000 registred commercial hybrids. They are sold as pot plant and cut flower production with an high economical value in international flower markets. Because of both considerations breeding in orchids is of most importace to generate new varieties and hybrid to correspond market needs. Manipulating ploidy levels in commercial and wild species is one of the possible way to create new genetic variability. Polyploidy may allow us to overcome sexual incompatibility barriers, to recover hybrid fertility by the generation of allopolyploids, which may have an improved pest and disease tolerance. Polyploids may also show an enhanced vigor and large general size. Actually, polyploid orchids have larger flowers, exhibit recurrent and longer blooming time. Cellular manipulations allow polyploid induction through the use of spindle formation inhibitors during mitosis. In our experiments we applied colchicine and amiprophos-methyl [APM; O-methyl-O-(4-methyl-6-nitrophenyl)-N-isopropyl-phosphorothioamidate] treatments to inhibit spindle organization on colture of *Dendrobium* protocorm like bodies (PLBs). PLBs are highly regenerative explants which can grow fast into liquid and solid medium *in vitro*. Their ploidy was analysed by flow cytometry which allow us for a rapid and soon screening of treatment effects. APM showed to be very effective in polyploidization at amounts which were $10^{-3}$ less concentrate than colchicine. The flow cytometry approach was able to show which treatments were the most active and less toxic to orchid cells, which in turn avoided excessive mutagenic treatments and in vitro propagation of true-to-type materials, thus reducing costs and experiment working time.

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## ENDOPLASMIC RETICULUM IS A TARGET OF CADMIUM TOXICITY IN THE CALCISPONGE

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Heavy metals are among the most dangerous and abundant inorganic environmental pollutants, arising from industrial discharges and mining practices. Unlike organic compounds, they cannot be degraded but accumulate throughout the trophic web, producing potential human health risks and ecological disturbance. Marine benthic filter-feeders are especially vulnerable to waterborne metals because they are able to process large amounts of water. We used the calcisponge *Clathrina clahtrus* as experimental model to study the effects of exposure to sublethal cadmium ($Cd^{2+}$) concentrations on the expression of GADD153, an endoplasmic reticulum (ER) stress marker. The presence of GADD153 immunoanalogue in *C. clathrus* was evidenced using Western blotting and confocal laser scanning microscopy. After 24 h of $Cd^{2+}$ exposure (1 and 5 μM), GADD153 was upregulated. Consistent with immunofluorescence staining, predominant protein bands appeared in the immunoblotting of treated cells in a dose-dependent manner whereas the corresponding labelled protein bands were only barely detectable in control cells. An increased expression of the ER molecular chaperone GRP78, followed by activation of ER-specific caspase-12, was also associated with increased GADD153. Results indicate that ER is one of the primary target organelles in $Cd^{2+}$-induced apoptosis and, therefore, ER stress proteins are key biomarkers of $Cd^{2+}$ toxicity in sponge cells.

## CHRONIC DISEASES AND CANCER PREVENTION – THE ROLE OF GENETIC DETERMINANTS OF DETOXIFICATION AND ENVIROMENTAL RISK FACTORS

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Individual susceptibility to cancer and chronic diseases results from several factors including inherited differences in metabolic capacity, environmental exposures to genotoxic agents, and nutritional habits.

The cellular systems for detoxification are essential to protect cells from damage by the exposure of various forms of environmental toxins and carcinogens.

Genetic polymorphisms that may cause lack of functional enzyme or low functioning enzymes, have been detected in many phase I, mainly cytochrome-P450-enzymes, and phase II detoxifying enzymes as glutathione-S-transferases (GSTs) and N-acetyltransferases.

In particular GSTs are a family of enzymes that exert a critical role in cellular protection against oxidative stress, pollutants, and toxic chemicals as heavy metals. In Cauca-

sian European populations in about 50% of individuals there is an absence of GSTM1 enzyme activity due to deletion of both copies of the GSTM1 gene (GSTM1 null genotype).

Epidemiologic studies have found that individuals with null genotype of this gene have an increased risk of cancer, neurodegenerative disorders, and skin manifestations. In our laboratory we perform routine genetic analysis of the most relevant metabolizing genes by real time PCR. In a group of 39 patients diagnosed for the mentioned pathologies and/or clinical tested high levels of heavy metals we found the overrepresentation of the GSTM1 null genotype (66%).

We propose genetic testing with the aim to individuate risk factors for diseases before they appear by modifying lifestyle and dietary habits in relation to unfavourable genetic constellations, but also to enable and sustain a specific therapy for known pathologies.

Genetic analysis are an invitation to actively play a role at the preservation of the own health.

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EMERGING INFECTIONS BY *FUSARIUM SPP.:* A CLINICAL CASE OF GANGRENOUS NECROSIS OF THE DIABETIC FOOT

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The fungus most frequently found in diabetic foot are linked to infection with dermatophytes, *Candida* spp. and nondermatophytic molds, such as *Fusarium* spp.

Fusariu*m* spp. is the causative agent of superficial mycoses and systemic hyalohyfomycosis, for this reason we focused on a case of *Fusarium* spp. infection in a patient with diabetic foot ulcers.

Clinical Case: Male patient of 67 years which was diagnosed diabetic gangrene of the left lower limb, admitted to the IX Division of General Surgery and Applied Biotechnology, SUN. Clinical examination showed trophic lesions infected of the left foot with diabetic gangrenous ischemia of the third and fourth finger. Suspecting an overlapping bacterial or fungal infection, scales skin samples were taken for a microbiological analysis. The bacteriological examination showed after 24h the development of colonies of *Staphylococcus aureus*, and only 2°-3° day was observed the development of a fast-growing filamentous fungus. The microscopic observation of the colony, by scotch test, showed hyaline septate hyphae of about 3-8 μm in diameter and the presence of cylindrical to oval one or two-celled hyaline microconidia. The presence of fusiform multicellular macroconidia(banana-like), singly or in clusters was highlighted only after 4°/5° days.

Conclusion: This case confirms the importance of close relation between clinicians and microbiologist and that a careful clinical evaluation and accurate diagnostic protocols allow a correct diagnosis and the early treatment of these infections.

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FLOW CYTOMETRIC ANALYSES OF HEPATOPANCREATIC CELLS IN TERRESTRIAL ISOPODS

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Due to their morphologic characteristics, which are related to the physiological condition, and their role in bioaccumulation of contaminants, hepatopancreas of terrestrial isopods (*Porcellio scaber*) has been thoroughly investigated by many authors. The aim of this study was to obtain from isopods hepatopancreatic cells, specimens able to be characterized by flow cytometry. For this goal we initially used different protocols, in order to establish the best one.

*Armadillidium vulgare*, a different species of terrestrial isopod, were collected at non contaminated area, (located near the Campus Scientifico of University of Urbino); after the dissection, the digestive glands were gently isolated from animals, and differently prepared for flow cytometric (FCM) analyses. Treatments were performed using different buffers and enzymatic solution. Finally the best protocol was chosen taking into account scatter signals, metabolic activity (CFDA positive cells) and viability (PI positive cells).

Scatter signals revealed the presence of two types of cells confirming the detection of the large B cells, referred as less numerous and the small S cells, more abundant, as shown in SEM and TEM data. Our preliminary results show that it is central to distinguish these different sub populations for their different sensitivity to stimuli (including treatments). In fact, the small cells appear to be less responsive and probably more resistant; furthermore their gating selection seems to be hardly standardized and reproducible. Finally, we found an higher percentages of PI positive cells into the large cells, qualifying these as an appropriate model to monitor the cellular response to different environmental stress.

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CELL LINE MAINTENANCE CONDITION INFLUENCES THE P-gp PROTEIN EXPRESSION IN Caco2 CELLS

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P-glicoprotein (P-gp, MDR1) is a transmembrane protein associated with multidrug resistance phenotype. Pharmacokinetic and pharmacodynamic studies based on *in* vitro cellular systems are used to predict the oral dosage of drugs that are often P-gp substrates. These measurements are clearly influenced by the P-gp amount present in each cell line used. In this work we demonstrate that the amount of P-gp of Caco-2 cell line, an established model system of intestinal absorption, is deeply influenced by cell culture conditions adopted during the cell line maintenance procedures. We compared the standard high density (HD) protocol of Caco-2 cell line maintenance with our recently published low density (LD) one. We measured the P-gp expression at both RNA (qRT-PCR) and protein (FACS analysis) level. We tested P-gp activity using the calcein uptake functional assay by FACS, and we measured cell sensitivity to both chronic and acute exposure of the cells to different P-gp substrates. The results demonstrated that cells grown in HD conditions express larger amounts of P-gp than the LD cultured cells, at both

RNA and protein level. Consequently, the analysis of drugs effect revealed that the HD cells were more resistant to the P-gp substrates. These finding underline the importance of a strict standardization of Caco-2 cell line maintenance protocol when this cellular model is used to predict the oral bioavailability of chemicals of pharmaceutical interest.

EFFECT OF EUPLOTIN C ON MEMBRANE PHYSICAL PROPERTIES OF THE CILIATED PROTOZOON *PARAMECIUM*

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We have previously demonstrated that euplotin C, a cytotoxic secondary metabolite produced by the protist ciliate *Euplotes crassus*, modulates $Ca^{2+}$ channel activity and swimming behavior in *Paramecium primaurelia*. This effect could be mediated by modifications of the physical properties of cell membranes related to the hydrophobic nature of the sesquiterpene molecule. To test this hypothesis we used laurdan, an environmentally sensitive dye with a red shift of the emission maximum when passing from a gel to liquid crystalline phase. These changes in the emission spectra may be quantified by calculating the so-called generalized polarization (GP). Furthermore, laurdan excitation with polarized light allows the measurement of anisotropy, a property related to membrane viscosity. Treatment of *Paramecium* cells with laurdan labeled not only the plasma membrane but also the intracellular membranes, food vacuoles and lipid droplets. GP and anisotropy values, measured in cell portions devoid of food vacuoles, were significantly higher in euplotin C-incubated cells than in controls. These results are consistent with the occurrence of an increased phospholipid ordering and a reduced fluidity of the membranes, following the euplotin C treatment. Our data suggest that euplotin C modulates swimming behaviour in *Paramecium* by interacting with the ciliary $Ca^{2+}$ channel functions through the reduction of cell membrane fluidity.

LABORATORY DETECTION OF GROUP B STREPTOCOCCUS AND EARLY NEONATAL STREPTOCOCCAL INFECTION

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Group B Streptococcus (GBS) is the leading cause of neonatal early onset infection. Even if the adoption of measure of prevention decreased the rate of early onset infection (from 1,8 to 0,34/1000 live birth in USA), case of GBS infection continue to occur. The objective of the study was to determine the incidence of early onset GBS infection in one year period in the UTIN of our hospital (from January to December 2010) and to determine the outcome of sepsis evaluation in infant born to mothers with GBS colonization. The charts of infants born to mothers with GBS colonization were reviewed for details of sepsis evaluation and management. The microbiology records were used to identify proven cases of GBS septicemia and meningitis in neonates born during the study period. Prenatal screening for GBS colonization in mother was found in 92,1%. During the study period 97 infants (8,9%) were born to mothers who were known to have GBS carriage at the time of delivery. Out of a total of 1078 live births in 1 year there were not infants with culture proven GBS septicemia. 11 out of these 97 infants had sepsis evaluation and received empirical parenteral ampicillin for at least 5 days. There were no cases of blood culture proven GBS sepsis among these 97 infants. However, there were 2 cases of probable sepsis. The incidence of early onset GBS sepsis was found to be low, only 2 case of suspected early onset sepsis. The strategy of sepsis evaluation and management according to CDC guidelines was found to be effective in preventing death and definite GBS septicemia in infants born to GBS colonized mothers.

## HEMATOLOGY

A FLOW CYTOMETRIC ANTIBODY PANEL TO RECOGNISE HIGH GRADE BCELL LYMPHOMA CARRYING MYC GENE REARRANGEMENT

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MYC rearrangement (MYC-R) with IgH or IgL genes is the molecular hallmark of Burkitt lymphoma (BL), but can also be found in a small proportion of diffuse large B-cell lymphoma (DLBCL) and in B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and BL (DLBCL/BL). Discrimination between DLBCL, BL and DLBCL/BL carrying MYC-R (MYC+) is highly relevant for the clinical management, because of the aggressiveness of these tumours and the need for proper therapeutic strategies. Several markers have been recently identified by either immunohistochemistry or flow cytometry (FC), which can help recognise MYC+ NHL. We used 6-colour FC to investigate the differential expression of some of these antigens, along with other markers included in

our FC routine panels, in a group of 17 high grade B-NHL (10 DLBCL and 7 BL) according to their MYC status. MYC-R was found by FISH (and, in 1 case, also karyotype) in all 7 BL and in 1 DLBCL. Expression of CD38 and CD81 was detected at significantly higher levels in MYC+ NHL compared to MYC-cases ($P = .01$ and $P < .01$, respectively). Conversely, CD22 was more likely to be down regulated in MYC+ cases. CD44, which was weak or absent in all MYC+ NHL, had a broad spectrum of expression in MYC-NHL, with bright staining in 3/8 cases. In addition, the immunoregulatory CD200 protein was constantly negative in MYC+ NHL but detectable, although weakly, in 5/9 MYC-cases. Interestingly, the only MYC+ DLBCL carrying a complex MYC-R showed high expression of CD38 and CD81, negativity for CD200 and moderate CD22 staining. These data confirm the immunophenotipic heterogeneity of DLBCL and indicate CD38 and CD81 as powerful markers that, together with CD22, CD44 and CD200, can be used in FC panels to recognise NHL with MYC-R.

## MULTIPARAMETER FLOW CYTOMETRY CAN DETECT OCCULT BONE MARROW INVOLVEMENT IN MYELOID SARCOMA

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Myeloid sarcoma (MS) is a tumour mass composed of myeloid blasts with or without maturation that occurs at an extramedullary site. MS may precede or coincide with acute myeloid leukaemia (AML) or represent acute blastic transformation of myelodisplastic syndromes (MDS). More rarely, MS may occur *de novo*, without a prior history of myeloid neoplasm, but with an eventual progression to AML. We report a recent case of a 80-year-old female patient with multiple hypochondria skin lesions and no history of haematological disease where an immunohistochemical diagnosis of MS was rendered. No marrow infiltration was seen in the biopsy. On the marrow aspiration, multiparameter flow cytometry (FC) revealed a rare population of myeloid cells (0.2%) partially overlapping with monocytes and granulocytes on the CD45 versus SSC plot and phenotypically identical to the extramedullary lesion (CD45+, CD33+, CD34-, CD117-, CD13-, CD4+, CD56+, MPO+). No MDS abnormalities were recorded by morphology or FC. Cytogenetic studies on the marrow aspiration yielded a normal karyotype. The FC finding of an occult neoplastic population suggests that in apparently *de novo* MS, concomitant medullary involvement might be already present, although morphologically undetectable. A sensitive approach like multi-colour FC with accurate gating procedures and appropriate antibody combinations should be concurrently employed to assess this possibility. Long-term follow up studies are needed in these cases to understand whether minimal medullary involvement should alert about a forthcoming progression to AML and then guide the choice and timing of treatment.

## PHENOTYPICAL AND FUNCTIONAL CHARACTERIZATION OF MESENCHYMAL STEM CELLS DERIVED FROM PATIENTS AFFECTED BY SCHWACHMANDIAMOND SYNDROME

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Shwachman-Diamond Syndrome (SDS) is an inherited marrow failure disorder characterized by varying cytopenias and pancreatic dysfunction. Neutropenia plays a crucial role in the occurrence of recurrent and severe infectious complications representing one of the major causes of death in SDS patients. The aim of our study is to better comprehend the marrow dysfunction occurring in SDS patients, by analysing the functional properties of bone marrow (BM)-derived mesenchymal stem cells (MSCs). MSC were obtained from 27 SDS patients. At the third passage of the culture, MSC were tested for the expression of specific surface markers, their ability to differentiate into mesengenic lineages, their capability to abrogate T cell proliferation and their capacity to prevent neutrophil apoptosis. MSCs derived from SDS patients (SDS-MSCs) displayed typical fibroblastoid morphology and expressed common MSC markers. These cells were able to differentiate into adipocytes and osteoblasts. In addition, SDS-MSCs drastically decreased the mitogen-induced lymphocyte proliferation. We also cultured neutrophils in presence or absence of MSCs. We demonstrated that SDS-MSCs were comparable to HD-MSCs in supporting the viability of neutrophils. SDS-MSCs were also able to produce high amount of IL-6, a cytokine involved in the protection of neutrophils from apoptosis. Genome wide gene expression analysis was carried out and preliminary results showed a SDS-MSCs specific profile, significantly different from HD-MSCs. In conclusion, we successfully isolated and characterized MSCs from SDS patients. Further studies are needed to better comprehend the functional and molecular features of SDS-MSCs, which are potentially involved in the hematological abnormalities typical of SDS patients.

## DOWNREGULATION OF MTOR AND P70S6K 2 IN PEDIATRIC TCELL ACUTE LYMPHOBLASTIC LEUKEMIA (TALL) IS CORRELATED WITH A POOR PROGNOSIS

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Background: Characterization of molecular alterations with prognostic impact in children with T-ALL may be use-

ful for an early identification of patients at high risk of failure. In the attempt to better characterize the role of the PTEN/AKT/mTOR pathway in pediatric T-ALL, we have evaluated the expression of each protein in this pathway's cascade and investigated its association with outcome.

Materials and Methods: We studied 23 children with T-ALL diagnosed at our Center and treated by three consecutive AIEOP protocols. We analyzed mRNA expression of PTEN, using RT-PCR. Using western blot analysis, we evaluated the expression of the following total and phosphorylated [AKT(S473)/(T308); GSK3β(S9); PTEN(S380); PDK1(S241); P70S6Kβ2(S371); mTOR(S2448); S6K(Ser235/236)/ (Ser240/241)] proteins. The association of these variables with the Event Free Survival (EFS) was assessed using the c2 test. A p value $\leq$0.05 was considered statistically significant. Furthermore, in order to measure the association level, the Relative Risk (RR) and the corresponding 95% Confidence Interval (95%CI) were calculated.

Results: RT-PCR analysis of PTEN revealed that one case did not show any product, whereas western blot analysis demonstrated that all patients showed total and phosphorylated PTEN proteins. Interestingly, we observed that total AKT protein was present in all the cases except one; the phosphorylated forms were detected as follows: AKT(T308) in 15 out of the 23 patients (65%), whereas none showed expression of AKT(S473). Surprisingly, we detected a statistically significant downregulation of total and phosphorylated mTOR and P70S6Kβ2 expression in eight, nine, ten and eleven out of 22 analysed patients respectively. Downregulation/absent expression of both total and phosphorylated P70S6Kβ2 had a statistically significant impact on EFS showing a higher risk of events, when comparing those downregulated with those exhibiting phosphorylated (RR: 2,75; 95%CI: 1,25-6,01) and total protein (RR 3,33; 95%CI: 1,29-8,59) respectively. Moreover, downregulation of mTOR(S2448) confirmed the same pattern of higher risk of events (RR: 2,77; 95%CI: 1,08-7,07) comparing those downregulated with those exhibiting expression of phosphorylated protein.

Conclusions: Our data have shown that the downregulation/absent expression of mTOR or P70S6Kβ2 is associated with a very poor outcome. Our data also identify new markers of aggressive and resistant disease, easily available at diagnosis.

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HLA AND HNA ALLOIMMUNIZATION STUDY IN LOMBARDY BLOOD DONORS

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Alloantibodies directed against both human leukocyte antigens (HLA) and human neutrophil antigens (HNA) have been associated with transfusion-related acute lung injury (TRALI). In this study we determined the frequencies of HLA and HNA alloimmunization in our region blood donors. The study has been performed in a cohort of 406 random blood donors, including 131 females and 275 males. HLA screening was done using LABScreen mixed, Luminex technique (OneLambda). HLA class I or class II positive sera from nulliparous women and from untransfused male were retested by flow cytometry using FlowPRA screening (OneLambda). Positive sera from untransfused males were further retested with FlowPRA High Definition (HD) class I assay in order to evaluate the antibody specificities. HNA antibodies screening was performed in two phases. Level 1 testing was an indirect GIFT on ten group 0 donor granulocytes performed on all studied samples. Level 2 testing used a larger panel of cells characterized for HNA antigens only on those samples resulted positive in level 1.

In this study 29% of the 131 female donors and 8% of the 275 males resulted to be alloimmunized. HLA class I and/or II antibodies accounted for 84% in female and 71% in male alloimmunized donors. With regard to HNA alloimmunization, after the first line screening, 6,8% of females had a positive GIFT compared to 2,5% of males. Moreover, anti HNA-1 antibodies have been identified in 6 alloimmunized males (anti-HNA-1a in 4 cases).

Comparing our data with literature, we report a higher portion of both male and female HLA immunized donors and a comparable level of HNA alloimmunization. The incidence of HLA and HNA alloimmunization could be helpful to better understand a common regional procedure for TRALI prevention.

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VALIDATION OF A NOVEL DIAGNOSTIC TOOL FOR JUVENILE MYELOMONOCYTIC LEUKEMIA BY FLOW CYTOMETRIC pSTAT5

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Juvenile myelomonocytic leukemia (JMML) is an aggressive myeloproliferative neoplasm of childhood characterized by excessive proliferation of monocytic and granulocytic cells. JMML hematopoietic precursor cells show *in vitro* hypersensitivity to granulocyte-macrophage colony stimulating factor (GM-CSF). Current diagnostic criteria for JMML are based on clinical features and laboratory findings. However, diagnosis is sometimes challenging. We and others have reported that *in vitro* response of JMML cells demonstrated a greater increase in the % of STAT5-phosphorylated cells (p-STAT5), by using a novel phospho-specific flow ctometric assay (Gaipa G et al., *Leukemia*, 2008; Kotecha N et al., *Cancer Cell*, 2008). Here we analyzed 19 JMML patients at diagnosis, 42 control subjects and 8 patients with suspected diagnosis of

JMML subsequently not confirmed. GM-CSF induced p-STAT5-responsive cells were identified within the CD34+/CD33+ subset. JMMLs and controls were compared at each dose using Wilcoxon's test in order to identify the best dose with lowest significative p-value after correction for multiplicity with a Bonferroni's method. We found that a threshold of 18.9 % of p-STAT5+ cells, after stimulation with 0.1 ng/mL GM-CSF ($p < 0.01$), was the best condition to discriminate JMMLs (n 8) from control subjects (n 27). This algorithm was then applied on an independent cohort of JMML (n 11), control subject (n 15) and patients with suspected diagnosis of JMML subsequently not confirmed (n 8). Sensitivity was 0.82 and a specificity was 1.0.

In conclusion JMML p-STAT5 hyper-responsiveness can be rapidly assayed by phospho-flow technology in the context of routine diagnostic work-up with high sensitivity and specificity, under appropriated technical standardization.

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## RESPONSE OF STEROIDREFRACTORY CHRONIC GRAFT VERSUS HOST DISEASE TO EXTRACORPOREAL PHOTOPHERESIS CORRELATES WITH THE DOSE OF CD3+ CD4+ LYMPHOCYTES HARVESTED DURING EARLY TREATMENT CYCLES

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Extracorporeal photopheresis (ECP) is the current therapy for steroid-refractory chronic graft versus host disease (cGVHD). Although the dose of nucleated cells collected during ECP has been suggested to play a role in treatment outcome, whether the latter correlates with lymphocyte subpopulation doses in ECP product is still unknown. Flow cytometry data obtained on each product from 11 ECP-treated cGVHD were compared with clinical response (NIH criteria). Absolute counts of lymphocytes and their subpopulations were determined using single-platform technology, with the CD3FITC/CD16-56PE/CD45PerCP Cy5.5/CD4PE-Cy7/CD19APC/CD8APCCy7 panel. For each cell population, (i) the mean dose harvested per procedure during the first 3 months, (ii) the cumulative dose harvested during the first 3 months, and (iii) the cumulative dose harvested until attainment of partial remission (PR) or last follow-up were evaluated. A total of 348 ECP were performed. Patients received a median of 29 (12–54) ECP during a median of 9.4 (4.2–26.1) months, and a median of 11 (8–14) ECP during the initial 3 months. PR was obtained in 8/11 patients. At Cox regression, harvesting at least a mean number of (i) 49x10^6/kg CD3+ lymphocytes or (ii) $^2$9x10^6/kg CD4+ lymphocytes during the first 3 months was associated with a higher PR probability ($p = .086$ and .093, respectively). Nevertheless, the achievement of at least one of these two targets significantly predicted PR by Mantel-Haenszel test ($p = .023$). No other parameter correlated with outcome.

Our preliminary data suggest that CD3+CD4+ cell evaluation in ECP could early predict PR. In view of the well known lymphocytolytic effect of corticosteroids, the negative impact of dosage/duration of previous immunosuppression on ECP efficacy could be explained.

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## THE ROLE OF CD200 EXPRESSION IN DIFFERENTIAL DIAGNOSIS AMONG NON HODGKIN LYMPHOMAS

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CD200 is a membrane glycoprotein, belonging to the immunoglobulin super family, expressed on a subset of T and all B lymphocytes (not on NK cells), dendritic cells and highly on central and peripheral nerve tissue. CD200 expression was found to be up-regulated in some Non Hodgkin Lymphomas (NHL) when compared with normal B cells and so useful in their diagnosis and therapy. In particular chronic lymphocytic leukemia (CLL) and hairy cell leukemia (HCL) express CD200 highly, allowing differential diagnosis between CLL and mantle cell lymphoma (MCL), HCL and splenic marginal zone lymphoma (MZL).We investigated CD200 expression on blood specimens (bone marrow and peripheral blood) of 46 normal controls, 105 patients with CLL, 22 with MZL, 7 with MCL, 9 with follicular lymphoma (FL), 7 with HCL, utilizing a BD FACSCanto II. CD200 expression was measured as mean fluorescence intensity (MFI) on B cell population for each control and patient specimen. Mean and standard deviation were then calculated on normal control group and on each group of NHL. When compared with normal B cells (286.33+/-142.86 MFI), CD200 expression resulted high in CLL (851.92+/−509.20 MFI) and HCL (769.14+/−582.62 MFI), almost normal on MZL (299.59+/−239.68 MFI and FL (238.89+/−175.47 MFI), and low in MCL (184.43+/−163.61 MFI). According to literature, we confirmed the utility of CD200 in differential diagnosis between HCL and MZL, especially in atypical HCL, and also between CLL and MCL. Comparing CD200 expression between NHL and normal B cells, we found that among MZL, FL and MCL, the latest has the lower expression. This finding may further help in differential diagnosis between MZL CD5+ and MCL. We need to validate our results on a bigger number of NHL.

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## THE ROLE OF CD19 AND CD27 IN THE DIAGNOSIS OF MULTIPLE MYELOMA BY FLOW CYTOMETRY: A NEW STATISTICAL MODEL

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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.192 bone marrow samples, from patients and controls, were enrolled in a study for routine diagnostic analysis of MM. A direct 7-8-color method was applied to study the immunophenotype of PC. Samples were labelled with fluorochrome-conjugated monoclonal antibodies (PacificBlue-FITC-PE-PerCP-APC-PeCy7-PacificBlue-APCCy7) to the following antigens: CD138, CD81, CD200, CD221, CD45, CD38, CD28, CD19, CD27, CD117, CD38, CD33, CD20, CD56, CD10, κ and λ light chains. CD19 and CD27 expression, when applied to our model, resulted in optimal concordance with histology. 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.

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IMMUNOLOGICAL PHENOTYPE AND FUNCTION ANALYSIS IN CHILDREN WITH FANCONI ANEMIA

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Introduction: Fanconi Anemia (FA) is considered a chromosomal instability syndrome with hypersensitivity to alkylating agents as principal diagnostic feature. Eighty percent of FA patients develop bone marrow failure with a high incidence of evolution in myelodysplasia and/or acute leukemia. FA patients generally do not have clinical or laboratory immunodeficiency although worsening of the peripheral blood cytopenia has been observed after infection. Therefore we aimed to evaluate the immunological status in FA patients before bone marrow transplantation. Method: In this work we analyzed the peripheral blood immunological phenotype in flow cytometry and serum immunoglobulin levels in 60 FA patients who had not been transplanted. In particular we studied CD3+, CD4+, CD8+, CD19+, CD16−/CD56+/CD3−, CD3+/DR+, CD3+/CD45+/RA+, CD3+/CD45+/RO+ and CD3+/CD4+/CD25bright+/FoxP3+.

Results: More than 50% of patients showed a reduced proportion of CD19+, CD3+CD45+ RO+ and NK while the proportion of CD3+, CD8+ and CD3+CD45RA+ was higher than age-matched normal values. T-regulatory cells studied in about 30 patients were lower than in healthy donors. Also IgM levels were lower than normal age matched samples in more than 50% of patients.

Discussion: These immunophenotype studies indicate immune dysfunction in FA patients. The increased level of CD8+ associated with decrease of T regulatory cells could explain the overexpression of cytotoxic cytokines and increased apoptosis seen particularly in hematopoietic stemcells. The low level of memory T cells seen could be due to the high chromosome instability and reduced DNA repair activity in these cells in general and thus in FA memory cells in particular (Michie et al, *Nature* 1992; Scarpaci et al, *Mechanisms of Ageing and development*, 2003).

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TEMSIROLIMUS, AN ALLOSTERIC mTOR INHIBITOR, IS SYNERGISTIC IN COMBINATION WITH CLOFARABINE IN ACUTE MYELOID LEUKEMIA

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Signaling through the PI3K pathway and its downstream effectors, AKT and mammalian target of rapamycin (mTOR), is abnormally activated in acute myeloid leukemia (AML), where it contributes to cell proliferation, survival, and chemotherapy resistance. Inhibiting mTOR signaling in AML blasts may enhance their sensitivity to cytotoxic agents. Preclinical data also suggest that allosteric mTOR inhibition with rapamycin impairs leukemic stem cell (LSC) function. In this study we assessed the combination of temsirolimus with clofarabine, which is a nucleoside analogue with potent inhibition effects on both ribonucleotide reductase and DNA polymerase. The combination of clofarabine and temsirolimus (CLO-TOR) was cytotoxic to a panel of AML cell lines and blasts from AML patients. Indeed, treatment with CLO-TOR induced a G1-phase cell cycle arrest and apoptotic cell death, as demonstrated by Annexin V/PI staining. CLO-TOR also induced apoptosis in an AML patient blast subpopulation ($CD34^+/CD38^-/CD123^+$), which is enriched in putative LSCs, as documented by quadruple staining and flow cytometric analysis of Annexin V-positive cells. In summary, the CLO-TOR combination could represent a valuable innovative treatment for AML patients, also in light of its efficacy against LSC.

## CD19-B CELL LYMPHOMA IN A PATIENT WITH MARGINAL ZONE LYMPHOMA (MBCL) AND SMALL CELLS LUNG CANCER (SCLC)

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Introduction: The concomitant presence of 2 different lymphomathous clones is an infrequent condition in a patient, but found in haematologic clinic. Instead, the existence in a patient of 2 B-cell lymphomatous clones, one of which without CD19, is rarely seen. We identified by cytometry in a SCLC patient 2 B-cell lymphomatous clones: one is ascribable to a MBCL, the other to a B-cell lymphoma with extraordinary loss of CD19.

Methods: We analyzed the immunophenotype of peripheral blood and bone marrow of the patient (male,70years) using the following monoclonal antibodies: CD45/CD3/CD4/CD8/CD19/CD5/CD23/CD20/CD22/CD 79b/CD38/FMC7/CD10/anti-sIg κ/anti-sIg λ.

Results: The immunophenotype showed the simultaneous presence of 2 clones belonging to B lymphocytic line: a clone was CD45+CD19+CD20+CD22+CD23-and with clonal restriction λ; the other clone was CD45+CD19-CD20+CD22+CD23-and with clonal restriction κ.

Conclusions: The analysis showed 2 B-lymphocytic clones: one has all the characteristic antigens of B lymphocytic line; the other, while presenting CD20 and CD22, lost CD19. In addition, CD19+ clone shows a clonal restriction λ, CD19-clone shows a clonal restriction κ. The 1st clone has the features of a MBCL; instead, the 2nd is lacking of the marker of B-cell line CD19, showing certainly an unusual phenotype, more undifferentiated and probably with more malignancy.

## EFFECT OF A GREEN TEA EXTRACT ON T REGULATORY CELLS IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA: PRELIMINARY DATA OF A CLINICAL TRIAL

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Regulatory T cells (Tregs) are considered to be key immunomodulatory cells of the immune system and are increased in chronic lymphocytic leukemia (CLL). Rai stage 0 identifies patients with early stage CLL for which there is no effective intervention at the present time and a "wait and see" policy is usually adopted. Several literature data reported that green tea constituents have antitumor effects on hematologic malignancies, including CLL.

We report preliminary data on a clinical trial in which a green tea extract, (Thè Verde®, Aboca), titled in epigallocatechingallate and caffeine, was given orally to patients with stage 0 CLL. Briefly, 7 patients were enrolled (mean age 56 years; range 29-75 years; 1 male and 6 females). An unitary dose of green tea extract (Thé Verde®, Aboca) was given daily for first month, followed by 3/2 dose for the next 5 months. At this time, only 4 patients were evaluable; 2 patients started therapy 2 months ago and 1 patient discontinued therapy after first month because of tachycardia. Of the evaluable patients, 2 showed a reduction of lymphocytosis and circulating Tregs as well; one patient showed a stabilization of lymphocytosis and a reduction of Tregs, while the last patient showed a little increase of lymphocytosis without Tregs modification. Only 2 patients showed trisomy 12 as single cytogenetic abnormality and only the non responding patient progressed after 5 months from the end of green tea administration, needing chemotherapy.

This data, despite limited in number, seem to indicate that green tea is able to modulate circulating Tregs in CLL patients with early stage of the disease, resulting in control of lymphocytosis and in prevention of disease progression.

## CYTOPLASMIC ACTIVE CASPASE-3 DETECTION BY FLOW CYTOMETRIC TO EVALUATE APOPTOSIS INDUCED BY *VERBENA OFFICINALIS* ESSENTIAL OIL AND ITS COMPONENT CITRAL IN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Caspase-3 is a key protease that is activated during the early stages of apoptosis. It is synthesized as an inactive proenzyme that is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease. We used flow cytometry to detect cytoplasmic expression of active caspase-3 in chronic lymphocytic leukemia (CLL) cells cultured with V*erbena* Officinali*s* essential oil and its component citral, a medicinal plant, also known as vervain, that we have previously showed to induce apoptosis in CLL cells. Briefly, cells from 8 patients (mean age 65 years; range 50–70 years; 5 male and 3 female) with previously untreated CLLwere incubated for up 6 hours at a density of $2{,}5 \times 10^6$/μL in RPMI 1640 at 37°C, 5% CO2, with a mixture of 90 μL of PBS and 0.1 μL of vervain essential oil and 9.9 μL of distilled water (to obtain vervain essential oil diluted at 1:100) or 10 μL of pure citral at the concentration of 1.9 mM. After incubation, cells were washed twice with cold PBS, then resuspended in Cytofix/Cytoperm solution at a concentration of $1 \times 10^6$ cells/0.5 ml

and incubated for 20 minutes on ice. Pelletted cells were then incubated with PE-conjugated polyclonal active caspase-3 antibody (BD Pharmingen) for 30 minutes at room temperature. Finally, after washing, resuspended cells were analyzed by flow cytometry (FACSCanto, Becton Dickinson).

All samples showed a high rate of apoptotic cells (active caspase-3 positive) after treatment with V*erbena* Officinali*s* essential oil (mean value 98.2 %; range 94–99%) and citral (mean value 93%; range 62–98%), as well. All but one patient were evaluated, by means of FISH, for cytogenetic anomalies with prognostic impact in CLL (+12, 17p13-, 13q14-, and 11q22-). Only one patient was found to carry deletion of 17p13, involving p53 gene. Studies on the molecular characterization of p53 genes are ongoing.

In conclusion, this data confirm results previously published by our own group on the ability of V*erbena* Officinali*s* essential oil and its component citral to induce *in vitro* apoptosis of CLL cells supporting further evidence that natural compounds

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HIGH SENSITIVITY OF FLOW CYTOMETRY IMPROVES DETECTION OF OCCULT LEPTOMENINGEAL DISEASE IN HAEMATOLOGIC MALIGNANCIES

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Cytomorphology (CM) of cerebrospinal fluid (CSF) fails to demonstrate malignant cells in up to 45% of patients in whom leptomeningeal disease is present. Flow cytometry (FC) is considered more sensitive than CM, but clinical implications of FC positivity/CM negativity are not established. CSF samples from 81 patients with haematologic malignancies were examined by CM and FC. The hae-matological diagnoses were: acute myeloid leukaemia (AML = 39), diffuse large B cell lymphoma (DLBCL = 17), B-lymphoblastic leukaemia (B-ALL = 13), T-lymphoblastic leukaemia/lymphoma (T-LL = 9), Burkitt lymphoma (BL = 2) and plasmacell leukaemia (PL = 1).Overall, 26 (32%) of 81 cases were FC positive; of these 26, 9 (35%) were also CM positive (FCpos/CMpos) while 17 (65%) were CM negative (FCpos/CMneg) ($p = 0.00002$). Of 17 FCpos/CMneg patients, 7 were affected with various forms of aggressive lymphoproliferative disorders and 10 with acute myeloid leukaemia (AML). Five patients (71%) of 7 with lymphoproliferative diseases developed overt central nervous system (CNS) disease whereas only 1 (10%) of 10 patients with AML experienced overt leukaemic meningitis. None of FCneg/CMneg patients experienced overt CNS disease ($p < 0.0001$). Among lymphoproliferative diseases, FCpos/CMneg patients showed a significantly shorter overall survival (OS) as compared to those FCneg/CMneg c($p = 0.008$). Conversely, AML patients who were FCpos had a similar duration of OS than those FCneg/CMneg. In conclusion, our data suggest that in lymphoid malignancies, FC significantly improves detection of leptomeningeal occult localization and predict overt disease, conversely in AML, FC positivity does not appear to have clinical significance, likely due to the use of ARAC based regimens and biology of disease.

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LEVEL OF MINIMAL RESIDUAL DISEASE (MRD) AND WHITE BLOOD CELL COUNT (WBCc) DISCRIMINATE DIFFERENT OUTCOMES AMONG ADULTS WITH FAVOURABLERISK ACUTE MYELOID LEUKEMIA

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Core binding factor acute myeloid leukaemia (CBF-AML) and AML with mutated Nucleophosmin (NPM) without FLT3-ITD mutation are currently regarded as favourable-risk AML, but recent findings suggest biological and prognostic heterogeneity of this AML subgroup. Our aim was to assess whether MRD detection was able to identify patients with increased risk of relapse. MRD was determined by multiparametric flow cytometry at the end of consolidation therapy. We evaluated 59 patients with de novo AML, enrolled in AML10/AML12 (n = 48) and AML17 (n = 11) EORTC/GIMEMA trials. Median age was 48 yrs (range 18–75), 36 were males and 23 females and 48 (81%) had white blood cell count (WBCc) $<50\times109$/L. Twenty-nine CBF-AML and 30 NPM-AML were evaluat-ed. Overall 24 patients (41%) relapsed. After consolidation, 21 patients underwent AuSCT, 13 AlloSCT and 23 did not received any transplant procedure. MRD positivity after consolidation (MRDpos) and WBCc $\geq 50\times109$/L were significantly associated to relapse ($p = 0.017$ and 0.0001, respectively). At 4 years, DFS for patients MRDneg vs MRDpos and with WBCc $<50$ vs $\geq 50\times109$/L was 70% vs 44% and 59% vs 21% ($p = 0.018$ and 0.011, respectively). At 4 years, CIR for patients MRDneg vs MRDpos and with WBCc $<50$ vs $\geq 50\times109$/L was 21% vs 55% and 33% vs 83% ($p = 0.005$ and $<0.001$, respectively). Therefore, we identified 3 different groups of patients: MRDneg/WBCc$<50 \times 109$/L, MRDpos/WBCc$<50 \times 109$/L and MRDpos/WBCc $\times$ 50x109/L with a 4-years DFS of 77%, 49% and 15%, respectively ($p = 0.001$) and a 4-years CIR of 12%, 47% and 90% ($p < 0.0001$). Among favourable-risk AML, the combination of WBCc plus MRD status identifies patients at higher risk of relapse for whom intensification by AlloSCT should be considered.

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DOES A MONOCLONAL T LYMPHOCYTOSIS EXIST LIKE MBL?

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Introduction: Flow cytometric (FC) immunophenotyping is the modality in the caracterization of undetermined lymphocytosis. Patients referred to our hospital for lymphocytosis were analyzed by FC. During 2010 we have analyzed 107 samples with lymphocytes between 4500 and 10000/mm3 and 15(14%) showed abnormal levels of T cells antigen such

as CD4 or CD8.We have further investigated TCD4 cells (CD4 from 3000 to 5000 $mm^3$) by using other T markers (like CD7 and CD26,whose absence is diagnostic for Sezary syndrome) to distinguish clonal from normal TCD4.

Methods: Cells from peripheral blood with TCD4 lymphocytosis were analyzed by FC. Samples with alterations were studied by molecular analysis and we have correlated the frequency of abnormalities in the expression in particular of CD7 and CD26 with TCRgamma gene clonal rearrangement.

Results: All positive for clonal T rearrangement phenotypic alterations showed and abnormalities were seen in the expression of one or more T markers.In detail CD7 was absent/dim in 50% and CD26 was undetectable in the entire 100% of patients.

Conclusions: Since a CD4+CD26-profile appears restricted to monoclonal TCD4 lymphocytes, CD26 expression might represent a useful tool to distinguish clonal T cells. Even if these CD4 proliferations might be defined as asintomatic lymphocytosis those patients should be monitored carefully, as represents a lymphocytic pattern with uncertain clinical outcome although presents the same immunophenotype of the Sezary syndrome.

## MONOCLONAL BCELL LYMPHOCYTOSIS (MBL): EXPERIENCE AT CASTELFRANCO VENETO HOSPITAL

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Introduction: Monoclonal B-cell lymphocytosis (MBL) indicates $<5\times10^9$ peripheral blood (PB) clonal B-cells/L with the phenotype of either CLL or another B-cell malignancy in individuals without clinical signs. In the past years different studies have suggested that it is a frequent phenomenon in the general population. We reviewed the presence of MBL in our specimens and particularly in the undetermined lymphocytosis analyzed in our laboratory by cytometry.

Methods: Routinely we have analyzed PB of patients referred to our hospital for analysis of lymphocytes with a panel that included complete blood count and immunophenotyping by cytometry protocols of a set of antibody for T, B, NK cells and for light chains and then we characterized the clonal B cells of the samples with light restriction.

Results: from 2009 in asymptomatic subjects aged >55 we have identified 70 MBL: of these, 43 were clonal B cells CLL-like ($CD5^+CD20dim^+CD23^+$), 7 atypical ($CD5^+CD20^+CD23^+$) and 20 CD5 neg. All, irrespective of the kind of MBL, had a mean age of 74 years. In the majority they freely repeated the test and only six (within one year) resulted in a clonal B lymphocytes $>5\times10^9$/L but without clinical signs again. In the 2011,one patient from CLL-like MBL with marker of risk (CD38+) turns into CLL and received specific therapy.

Conclusions: our review confirms as MBL is a hematologic condition with a mild course and apparently has minimal potential to progress of CLL

## PAX5/TEL FUSION PROTEIN AFFECTS TRANSCRIPTION PROFILE OF PREBI CELLS, CAUSING AN OPPOSITE DOMINANT EFFECT ON PAX5 PATHWAY, A REDUCED ADHESION CAPACITY AND A BLOCK OF B CELL RECEPTOR ASSEMBLY

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PAX5 is a transcription factor essential for B-cell development and it is a frequent target of genetic abnormalities in B-cell precursor ALL. The role of these lesions is poorly understood. Among its translocations, *PAX5/TEL* fusion gene is the most frequent; previously, we showed that the PAX5/TEL caused resistance to apoptotic effects of TGFbeta1 and enhanced migration to CXCL12. The aim of the study was understanding how PAX5/TEL affects the transcription process. We analyzed gene expression profile (Affymetrix) of murine pre-BI cells transduced by MIGR-PAX5/TEL and sorted by GFP (FACSAria). Validation of Differentially Expressed Genes (DEGs) has been performed by RQ-PCR and FACS analyses. PAX5/TEL significantly affected the transcription process: 61% of DEGs were down-and 39% up-regulated. In particular, genes normally activated by PAX5 were repressed by PAX5/TEL and, vice versa, genes physiologically repressed by PAX5 were activated by PAX5/TEL. Moreover, PAX5/TEL modulated molecules related to transcription, phosphorylation, B cell receptor signaling and adhesion. Here, we demonstrated that PAX5/TEL cells showed a significant reduction of adhesion capacities. Furthermore, we assessed the repression of numerous key molecules fundamental for BCR signaling, such as CD19, *BLNK*, *CD79a* CD22, *IRF4*, SLAMF6, *IKZF2*, *IKZF3*. Indeed, PAX/TEL cells were completely unable to complete IgM assembly and expression (FACS analysis). These results sustain the role of PAX5/TEL as an aberrant transcription factor, with a dual effect on PAX5 pathway, that we defined *opposite dominance*. The biological consequences of this aberrant activity are the impairment of B cell receptor and the reduced adhesion capacity, processes involved in leukemia transformation.

## CONCORDANCE OF FLOW CYTOMETRY AND RQPCR IN MINIMAL RESIDUAL DISEASE DETECTION IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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In the AIEOP-BFM ALL2000 trial, childhood-ALL patients were stratified mainly according to minimal residual disease (MRD), as detected by PCR amplification of immunoglobulin and T-cell receptor gene rearrangements. In this context, we also applied flow-cytometry (FCM) for prospective MRD measurements at days 15, 33, and 78 of induction therapy on 3565 samples (1547 patients), with the aim to compare the performances of the two techniques. The overall concordance was 80%, but different results were observed according to time point. Most discordances were found at day 33 (concordance rate 70%), in samples that had significantly lower MRD levels than concordant cases. However, the difference in sensitivity was not due to divergences in starting materials (total *versus* mononucleated cells), but rather to cell input number. At day 33, cases with MRD below or above the 0.01% cut-off by both methods showed a very good (5-year-EFS 91.6%) and a poor (5-year-EFS 50.9%) outcome respectively, whereas discordant cases showed similar EFS (around 80%). This finding suggests a potential complementary role of the two technologies in optimizing risk stratification in future clinical trials. However, either FCM or PCR can be used in MRD-based treatment protocols depending on the available expertise, resources and specific protocol design.

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A SINGLETEST SIXOR EIGHTCOLOUR FLOW CYTOMETRY ASSAY TO MONITOR CLL CELLS DURING THERAPY

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Monitoring of residual disease in CLL is increasingly becoming part of clinical trials for assessing the outcomes of therapy. Multiparameter flow cytometry (FC) is highly sensitive in recognising and quantifying CLL cells relying on their aberrant immunophenotype profile (i.e. CD5 expression and CD20, CD22, CD79b and CD81 down regulation). We aimed to ascertain the accuracy of these disease-specific markers in detecting malignant cells with the purpose to identify a sensitive and specific antibody combination easy to use in diagnostic routine practice. A series of 384 samples (245 PB and 139 BMA) obtained from 143 CLL/SLL patients at onset and follow up was regularly screened for CD5, CD19, CD20, CD22 and CD81 expression by 3-, 6-or 8-colour (C) FC. In 125 samples CD79b was also investigated, while k and λ clonality was assessed in 147. High concordance (97.2%) between low CD20, CD22, CD79b and CD81 versus k/λ chain restriction was found (sensitivity and specificity 96.4% and 100%, respectively). The only 4 discordant cases were atypical CLL showing strong CD20, no CD23 or low FMC7 expression and high levels of CD22, CD81 or CD79b. The introduction of 6-C FC allowed the use of a single-tube test containing the disease-specific CD81/CD22/CD19/CD20/CD5/CD45 combination. By this test, minimal residual disease detection could be improved from values around 0.1% (for 3-C FC) to values close to 0.01%. Gating strategies and extensive phenotypic characterisation are being currently enhanced by 8-C FC using a highly specific CD81/CD22/CD19/CD33/CD79b/CD20/CD5/CD45 combination. By multicolour analysis, a valid single-tube test can therefore be employed that quickly and easily provides accurate identification and enumeration of CLL cells at onset as well as follow up.

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BCELL NEOPLASMS WITH LACK OF CD19 BUT CyCD79a EXPRESSION

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From studies of neoplastic cells it was initially suggested that CD19 is the first B-cell antigen to appear on lymphoid progenitors, thus heralding lineage-commitment, but other data demonstrated that cyCD79a seemed a good candidate antigen to specifically delineate early B-lineage affiliation. Investigation of normal human bone marrow led to the conclusion that, on physiologic TdT+/CD34+ precursors, CD19 and CD10 appear essentially at the same time. CD10 is the upstream marker in early B cell ontogeny, but it is not B cell specific.

By multiparametric flow cytometry analysis we observed bone marrow cells of two patients affected by B cell neoplasms that lack CD19 but express cyCD79a.

The patients included a ALL "common" and a lymphoplasmacytic lymphoma/Waldenstrom Macroglobulinemia. In the present study, we analyzed B-antigen expressions (CD19, CD20, CD10, CD22, CD23, CD24, cyCD79a) and we focused on the analysis of CD19 and cyCD79a mole-cules. To define atypical expression, we detected CD19 with MoAbs coniugated by FITC, PE, APC and Pe-Cy7. Our data document for the first time that many of those TdT+ progenitors that lack CD19 express cyCD79a in addition to CD10, which corroborates the concept that these cells are committed to the B lineage and which implies that cyCD79a precedes CD19 in the earliest differentiation events of B-cell ontogeny.

## AUTOLOGOUS BONE MARROW CELLS USED FOR TISSUE REGENERATION

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The haemopoietic progenitors can be detected in bone marrow, in human umbilical cord blood and in mobilized peripheral blood after myelosuppressive chemotherapy e/o growth factors administration. The stem cells are used for transplantation and are able to reconstitute haemopoiesis. Recent studies suggest that bone marrow stem cell concentrates are used in many diseases requiring tissue regeneration. We describe two orthopedic patients with osteonecrosis of femoral head, treated with autologous bone marrow grafting obtained from the iliac crest. The aspirated marrows were reduced in volume by centrifugation to 6–7 ml in order to preserve only mesenchymal stem cells. The implantation was performed by topical application. The aim of this study was to characterize the main cell populations by flow cytometry analysis (FACSCanto II). We tested the MoAbs CD14FITC, CD117PE, CD34PECy7, CD33APC, CD45APC-Cy7. Generally the stem cell is lineage negative and expresses CD34. Previous studies had shown that CD34 expression reflects the activation/kinetic state of hemopoietic stem cells and that it is reversible, in fact mobilized stem cells express CD34, return to the bone marrow and become CD34negative when the bone marrow achieves steady state. On the contrary, we identified early hematopoietic progenitor population doesn't express CD34, shows low CD45 and expresses CD117 (c-kit receptor), while CD117expression declines with terminal myeloid and erythroid differentiation. Therefore, only CD34negative/CD117positive bone marrow cells are capable of long-term hematopoietic reconstitution. This population is able to improve cartilage reconstitution, therefore, it is likely to be first choice treatment in stages I-II of osteonecrosis of the femoral head.

## CD64 EXPRESSION ON GRANULOCYTES FROM PRETERM AND TERM NEWBORN INFANTS

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The bacterical infections represent an important cause of mortality in neonatal medicine. The expression of CD64 on granulocytes can be a useful tool as a diagnostic marker for sepsis in neonates. The presence of CD64 is increased from an almost negligible to a marked level on neutrophils in patients with bacterial infections, due the influence of inflammatory cytokines. Granulocyte CD64 expression from preterm and term newborn infants was analyzed by multiparameter flow cytometry. We studied 47 neonates: 42 preterm infants and 5 term newborn infants. In these patients we analyzed the granulocytes on pheripheral blood at birth by a multiparameter flow cytometry analysis. We employed a FACSCalibur (BD). The combination of monoclonal antibodies included CD16 CD11b CD13 CD64 CD14 CD45. We have compared CD16/CD13 CD16/CD11b CD64/CD11b maturity profiles. We focused our attention on CD64 expression. Interestingly, CD64 resulted always over-expressed, significantly increased and correlated with adverse prognosis and mortality in 16/42 preterm infants as compared to 5 term newborns. By contrast, there were no significant differences on the other studied antigens in these two groups. The study of distinctive maturity profiles and CD64 expression by flow cytometry shows important vantages: use of little quantity of blood and quickness of answer. In particular, the CD64 expression seems to be a useful marker of early diagnosis for early or late on set sepsis in neonates.

## EPCs CIRCULATING LEVELS IN ESSENTIAL HYPERTENSION

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Endothelial progenitor cells (EPCs) contribute to the integrity of vascular angiogenesis, acting in protection from atherosclerosis. Their number and functional capacity are reduced in patients with essential hypertension (EH). Endothelial dysfunction mediated by inflammatory cytokines and arterial stiffness (AS) represent an early stage in the development of atherosclerosis and is important indicators of subclinical damage. We evaluated the association between AS, inflammatory cytokines and circulating EPCs in patients with EH.

Sixty untreated patients, suffering from hypertension, with-out cardiovascular complication or diabetes mellitus, have been enrolled in this study. Functional AS (PWV) and the circulating cytokine network (Biochip array, Randox, Labs.) have been evaluated. On the same time the pool of circulating EPCs was determined by flow cytometric analysis of surface antigens: CD34, CD133 and VEGFR2 (KDR). This analysis has been performed establishing an immunological gate on total lymphocytes, by the acquisition of 20.000 events, over a total of 80-100.00.

The data showed that PWV is inversely correlated with circulating EPCs and strong directly correlated with the levels of TNF-α, IL-6, but less with IL-1β. The levels of EPCs are inversely correlated with IL-6, TNF-α and IL-1β. The stepwise multiple regression analysis showed that levels of EPCs are the strongest independent predictor of PWV.

Circulating levels of EPCs, as well as those of some inflammatory cytokines, are closely related to AS. In the presence of elevated levels of inflammatory cytokines, especially of TNF-α, low number of EPCs, associated with an increase of AS, promotes the onset and progression of atherosclerosis.

## ANALYSIS OF HEMATOPOIETIC PROGENITOR CELLS IN EARLY PRETERM CORD BLOOD BY THE USE OF ALDEHYDE DEHYDROGENASE AND MULTIDIMENSIONAL FLOW CYTOMETRY

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Recently, we demonstrated by flow cytometry (FCM) that early preterm cord blood samples (hEPCB) are enriched for immature hematopoietic stem cells (HSC) respect to the full term counterpart (hTCB).

Now, we want to investigate HSC by FCM on the basis of Aldehyde Dehydrogenase (ALDH) activity and CD34 surface expression in 10 hEPCB (17-27 weeks of gestation) and 9 hTCB (36–41 weeks of gestation) samples. We disclosed three subpopulations of HSC: $ALDH^+CD34^+$, $ALDH^+CD34^-$ and $ALDH^-CD34^+$. All these three subgroups were sevenfold more represented in hEPCB as compared to hTCB samples, being the median % of 1.85 *vs*. 0.27 for $ALDH^+CD34^+$, 0.6 *vs*. 0.07 for $ALDH^+CD34^-$and 0.55 *vs*. 0.07 for $ALDH^-CD34^+$ ($p < 0.01$). Moreover, all these subsets were screened for lineage (CD45, CD5, CD13, CD33, CD19, CD71, CD235a) and non-lineage associated antigens (CD26, CD34, CD38, CD117, CD133 and CD338). In both kind of cord blood specimens, $ALDH^+CD34^-$and $ALDH^-CD34^+$ HSC were enriched for myeloid and lymphoid committed progenitors, respectively. This finding was particularly apparent in hTCB group, which significantly manifested a more pronounced cellular heterogeneity.

As for $ALDH^+CD34^+$ subset, the median % of $CD133^+$ cells was 65.2 and 78.2 in hEPCB and hTCB samples, correspondingly. Moreover, $CD38^-$cells were 41.9% and 34.4% in 'early' and 'term' samples, respectively. Of note, in case of hEPCB, circulating $ALDH^+CD34^+$ cells displayed features typical of fetal liver immature HSC due to high expression of CD33 (median % was 97.9% in hEPCB *vs*. 57.6% in hTCB, $p < 0.001$).

Taken together, these data confirm the undifferentiated profile of hEPCB cells, while adding new insights regarding their more homogeneous composition, particularly as concerns the predominance of fetal-like cells in $ALDH^+CD34^+$ fraction.

## MULTICENTRE VALIDATION OF A REPRODUCIBLE FLOW CYTOMETRIC SCORE FOR THE DIAGNOSIS OF LOWRISK MYELODYSPLASTIC SYNDROMES: RESULTS OF A EUROPEAN LeukemiaNET STUDY

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The pathological hallmark of myelodysplastic syndromes (MDS) is marrow dysplasia, which represents the basis of the WHO classification of these disorders. The WHO proposal has raised some concern regarding minimal diagnostic criteria particularly in patients with normal karyotype without robust morphological markers of dysplasia, such as ring sideroblasts or excess of blasts.

We aimed to develop and validate a flow cytometric score (FCM-score) for the diagnosis of low-risk MDS. CD34+ cell compartment is peculiarly perturbed in MDS and therefore CD34-related parameters are good candidates for the identification of diagnostic markers for these diseases. Four reproducible parameters were analyzed: percentage of marrow CD34+ myeloblasts and B-cell progenitors, myeloblast CD45 expression and granulocyte side scatter (SSC) value. Study enrolled subjects with peripheral blood cytopenia from 6 european institutions and comprised a "learning cohort" (including 538 patients) to define the score and a "validation cohort" (including 258 patients) to confirm its diagnostic value. With respect to non-clonal cytopenias, MDS presented increased myeloblasts ($P < .001$), lower B-cell progenitors count ($P < .001$), aberrant CD45 expression ($P < .001$) and reduced SSC ($P < .001$). Defined reference ranges for each cytometric parameter were as follows: percentage of CD34+ myeloblasts <2%, percentage of CD34+ B cells >5%, Gra/Ly SSC ratio >6 and 4 <Ly/Mbl CD45 ratio <7.5. The FCM-score was defined by combining the 4 parameters in a general logistic regression model: based on regression coefficients a score value of 1 was assigned to each variable and a diagnosis of MDS was formulated in presence of a FCM-score value >=2. A repeated measure analysis of variance showed that the between-and the within investigator did not affect significantly the results of cytometric analysis among different laboratories. In the learning cohort a correct diagnosis of MDS was formulated in 198/281 cases (sensitivity 70%), while18 false-positive results were noticed among 257 controls (specificity 93%). Sixty-five percent of patients without specific markers of dysplasia were correctly classified. Based on the

FCM-score cut-off value, the positive predictive value (PPV) and negative predictive value (NPV) were 92% and 74%, respectively. The likelihood ratio [LR, defined as sensitivity/(1 -specificity)] was 10, without significant differences between patients with and without specific markers of dysplasia. A high FCM-score value was associated with multilineage dysplasia (P=.001), transfusiondependency (P=.02), and poor risk-cytogenetics (P=.04). The diagnostic value of FCM-score was tested in an independent cohort of 258 patients, showing comparable sensitivity (69%) and specificity (92%).

Overall our results indicate that immunophenotyping may consistently help establish the diagnosis of MDS, especially when morphology and cytogenetics are indeterminate.

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## INFANT BCELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA: COMPARISON OF IMMUNOPHENOTYPES AT DIAGNOSIS AND RELAPSE

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Acute lymphoblastic leukemia (ALL) in infants is a distinct subtype of ALL with unique biological features and poor outcome. Although immunophenotype of infants B-cell precursor ALL (BCP-ALL) is well described, significant changes in antigen expression could occur in case of relapse.

Aim: Comparison of infant BCP-ALL immunophenotypes at diagnosis and relapse.

Methods: Immunophenotyping at diagnosis was performed in 93 infants aged from 2 days to 11 months. Pared diagnosis and relapse samples from 27 patients (pts) with BCP-ALL were studied. In 23 pts (85.2%) *MLL*-rearrangements involving different partner genes were detected, 3 pts (11.1%) had germline *MLL*, for 1 pt (3.7%) *MLL*-status was unknown.

Results: 19 pts (70.4%) were diagnosed as BI-ALL, 5 pts (18.5%) as BII-ALL, 3 pts (11.1%) as BIII-ALL. At relapse 3 BII cases converted to BI, in 1 BIII case loss of cytoplasmic μ-chain (cyμ) was detected and it became BII, 2 BIII pts lost CD10 (in one case cyμ also disappeared), while in 1 BI case cyμ was gained. Additionally 1 pt, BI CD7(+) at diagnosis, relapsed as acute bilineage leukemia (BI CD7(-) + AML). 1 out of other 2 BI CD7(+) pts also lost CD7, while this antigen was gained in 3 BI and 1 BIII cases. In 5 cases blasts lost NG2; 2 pts, including one with germline *MLL*, became NG2-positive. CD15 and CD65 myeloid antigens, frequently expressed by *MLL*-rearranged BCP-ALL, were gained in 2 and 4 cases respectively, and 4 pts lost CD65. Stem cell markers, CD34 and CD133, were gained in 4 and 5 and lost in 2 and 2 cases correspondingly. 7 and 5 pts became CD22(+) and CD20(+) respectively. Marked immunophenotypical changes occurred in both *MLL*-rearranged and *MLL*-germline pts.

Conclusion: Significant immunophenotypical changes occur in infants BCP-ALL between diagnosis and relapse.

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## ABERRANT ANTIGEN EXPRESSION (AAE) AS A TOOL TO IDENTIFY PATIENTS WITH CLONAL MYELODYSPLASIA

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Myelodysplastic syndromes (MDS) are a group of clonal disorders of the bone marrow characterized by peripheral cytopenias. In this study, we addressed the question whether flow cytometry (FCM) was able to identify a series of phenotypic aberrancies in CD34+ cells taken from MDS bone marrow samples. FCM has proved to be one excellent method for detecting submicroscopic populations of acute myeloid leukemia blast cells. These results have been obtained based upon the identification of antigen combinations specifically expressed on leukemic cells, but not on normal cells, with a sensitivity of $10^{-4}$. Now, we have applied the same technique to detect aberrant antigen expression (AAE) in CD34+ cells taken from bone marrow samples drawn from patients with MDS. The antibody panel included five six color combinations: 1) CD15/ CD117/ CD45/ CD33/ CD34/ HLA-DR, 2) CD5/ CD7/ CD45/ CD13/ CD34/ CD4, 3) CD2/ CD11b/ CD45/ CD56/ CD34/ CD16, 4) CD19/ CD13/ CD45/ CD33/ CD34/ HLA-DR 5) CD71/ CD33/ CD45/ CD56/ CD64/ CD14. The identification of atypical cells allowed us to formulate the hypothesis of a clonal MDS diagnosis. We focused on two particular aspects of AAE immunophenotype: i) mixed-lineage (myeloid+lymphoid) profiles and ii) maturation asynchronisms (early+late specificities). We studied 40 consecutive MDS patients. Taken together, results show that 18/40 patients (45%) clearly dysplayed an AAE phenotype. Observed immunophenotypic aberrancies were the expression of a lineage infidelity marker (CD5, CD7, or CD56) the coexpression of an 'early' and a 'late' antigen (CD117/CD15 and CD34/CD11b), the loss of CD45 and the loss of the myeloid antigen CD33. In conclusion, FCM may add significantly to consolidated diagnostic parameters in identifying clonal MDS patients.

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## ENDOTHELIAL PROGENITOR CELL MOBILIZATION INTO PERIPHERAL BLOOD FOLLOWING TREATMENT WITH GCSF AND PLERIXAFOR

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There is compelling evidence that circulating endothelial progenitor cells (EPCs) exist in adult humans that reside

in the bone marrow and are able to home to sites of vascular injury and stimulate angiogenesis. A feasible strategy for stem cell therapy is to stimulate the mobilization of progenitor cells from the bone marrow into the circulation. This approach has been clinically established using G-CSF (and more recently CXCR4 antagonists, such as plerixafor) to mobilize hematopoietic progenitor cells (HPCs) for allogeneic transplantation. We aimed to analyse EPC mobilization into peripheral blood after treatment by G-CSF alone and combination with plerixafor. The study enrolled 22 healthy donors for allogeneic transplantation who received G-CSF 10 mcg/kg for 4 days and 16 patients with hematological malignancies receiving G-CSF followed by plerixafor injection (0.24 mg/kg). EPCs were defined as endothelial colony-forming cells (ECFCs, according to Ingram et al, Blood 2007). In healthy donors receiving G-CSF no significant difference was noticed between the frequency of circulating ECFCs at baseline (0.19 $\times$ 107 MNCs $\pm$ 0.44) with respect to both circulating ECFC at day of leukapheresis (0.0x107 MNCs $\pm$0.29, P = .36) and ECFC frequency in leukapheresis product (0.13x107 MNCs $\pm$ 0.36, P = .48). In 6 cases we performed a daily evaluation of ECFC frequency, and we found that circulating ECFC level did not significantly change during G-CSF treatment (P = .43). The frequency of circulating EPCs was also evaluated by flow cytometry as percentage of CD45-CD34+ cells in all MNCs. In keeping with ECFC assay results, with respect to baseline, no significant modification of the percentage of CD34+CD45-was noticed nor during G-CSF administration (P = .12) either in the leukapheresis product (P = .22), while the proportion of CD45+CD34+ HPCs significantly increased during treatment (P = 0.004). Moreover, we evaluated CXCR4 expression on both circulating hematopoietic CD34+CD45+ and endothelial CD34+CD45-cells. The percentage of CD34+CD45+CXCR4+ cells significantly decreased from day 0 to day of leukapheresis (P = 0.037), while no significant modification was noticed on the percentage of circulating CD34+CD45-CXCR4+ cells (P = 0.13). We next considered patients receiving G-CSF and plerixafor for HPC mobilization. The observed frequency of circulating ECFCs at baseline (0.64 $\times$ $10^7$ MNCs $\pm$ 1.0) was not significantly different with respect to circulating ECFC at day of leukapheresis (0.41 $\times$ $10^7$ MNCs $\pm$ 1.0, P = .78). Similarly, the daily evaluation of circulating EPCs by flow cytometry did not show significant modification on CD34+CD45-cell percentage with respect to baseline nor during treatment (P = .35). Finally, we analysed the percentage of both hematopoietic CD34+CD45+ and endothelial CD34+CD45-cells expressing CXCR4 at day before and after plerixafor administration. The percentage of CD34+CD45+CXCR4+ cells decreased after plerixafor administration (p = .043), while the percentage of CD34+CD45-CXCR4+ cells did not significantly change (P = .14).

In summary, despite its feasibility, G-CSF alone or combined with plerixafor fails to mobilize ECFCs into peripheral blood. We observed no significant modification of CXCR4 expression on circulating EPCs during treatment with G-CSF and plericxafor. Alternative strategies are warranted to obtain adequate EPC number to stimulate therapeutic angiogenesis.

## MONOCENTRIC STUDY OF EARLY IMMUNE RECONSTITUTION IN 29 PEDIATRIC PATIENTS UNDERWENT ALLOGENEIC CORD BLOOD TRANSPLANTATION: CORRELATION WITH POSTTRANSPLANT OUTCOME

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Introduction: many studies demonstrated that transplantation using umbilical cord blood stem cells (UCBT) is now an established life-saving treatment for children with hematological malignancies (HM) or inborn errors of metabolism (IEM). UCBT offers some advantages over Bone Marrow or Peripheral blood stem cells i.e. easier procurement and availability, lower incidence of GvHD. However, few cell dose and slow hematopoietic and immune recovery frequently facilitate graft failure(GF) or relapse(RE) of primary disease. Here we report the results, collected in the last 5 years in our HSCT Unit, about a study of immune reconstitution and chimerism (Ch) at early times post UCBT, in relationships with the follow-up of patients.

Patients and Methods: we studied 29 pts with HM (12) or IEM (17), all received myeloablative conditioning and 8.6$\times$10(e)7/Kg NCC. For each pt we analysed peripheral blood at engraftment (GB $\geq$ 1000) and day 30. Immunophenotyping by 6-color flow cytometric analysis was performed with a large panel of mAbs to characterize T-, B-and NK-cell. Ch was detected through PCR amplification of nine STRs.

Results: according to published values of lymphocyte subsets in children we identified two groups of pts at time +30 (median values) Tcells > 65% (66.1-97): 11 pts CD4/CD8 = 0.1, CD8CD45RO+ = 74.8%, NK = 12.4%, Ch 95%; outcome 7/11 (63%) GF or RE Tcells < 65% (0.0-60.8): 18 pts CD4/CD8 = 0.9, CD8CD45RO+ = 19%, NK = 61.1%, Ch 100%; outcome 2/18 (11%) RE post day +180.

Conclusions: our experience shows that, despite the high proportion of immature T cells usually found in UCB, about 1/3 of pts exhibits uncommonly rapid recovery of mature T subsets. This occurrence is not related, however, to improvement in immunity but to undesirable failure of transplant even with donor Ch prevalence.

## B MEMORY CELLS IN MULTIPLE MYELOMA

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Introduction: Multiple myeloma (MM) is a neoplasm with accumulation in the bone marrow of a Plasma cells clone. We investigated the B memory compartment of patients with MM, using the Bm1-Bm5 and CD27/IgD classification.

Materials and Methods: Fifteen MM bone marrow (BM) and peripheral blood (PB) samples of patients from Hema-

tology Division of Civic Hospital of Palermo were studied. Phenotypic study was carried out using the following panels: 1) CD24FITC/IgDPE/CD45PercP-Cy5.5/ CD38+ CD138APC/CD20APC-H7/CD19PacificBlue; 2) CD27FITC/ CD56PE/CD45PercP-Cy5.5/ CD38+CD138APC/CD20APC-H7/CD19PacificBlue. Acquisition of these samples were performed with CyAN ADPTM and analyzed with analysis software Kaluza (Beckman Coulter).

Results: According to the expression of CD27 and IgD, we have observed that: naïve B cells are the most representative population (55%) in PB samples, but in BM there is a greater presence of the population late memory (52,5%). In PB and BM samples, according to the Bm1-Bm5 classification, B cells are distributed between Bm5 early (BM = 17.5%; PB = 3.18%) and Bm5 late (BM = 35.3%; PB = 34.9%); as well as naïve B cells are included in Bm1 (BM = 31.9%; PB = 50.9%) and Bm2 (BM = 8.52%; PB = 6.29%) gates. In BM Plasma cells population it was noted that the CD19-CD56+ population when represented with a percentage >10% and <10% corresponds to the average of 61 % and 47% late memory B cells respectively.

Conclusion: In recent years several studies have focused on the phenotypic characteristics of memory B cells. This has provided a starting point for the MFC analysis of B lymphocytes antigen-dependent maturation in BM and PB samples, and their relationship with the pathological counterpart of Plasma cells population.

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FLOW CYTOMETRIC ANALYSIS: TWO DIFFERENT WAYS TO WORK

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Introduction: In this work two methods are compared using the equipments available in our laboratory, the BD FACSCantoIITM cytometer (Becton-Dickinson), through which will be analysed the samples labeled with monoclonal antibodies supplied by BD, and the CyAN ADPTM (Beckman Coulter) cytometer through which will be acquired samples previously labeled with the Solastra B Lineage (Beckman Coulter). Materials and Method: Thirty-one peripheral blood (PB) samples and nineteen bone marrow (BM) samples of patients from Hematology Division of Civic Hospital of Palermo were studied. Samples were stained with Solastra B Lineage kit, BL1 and BL2; and with the BD monoclonal antibodies: CD10PE/CD19PercP-Cy5.5/CD20PE -Cy7/CD38APC/CD45APCH7, KappaFITC/LambdaPE/ CD19PercP-Cy5.5/CD5APC/CD45APCH7. Acquisition was performed with CyAN ADPTM cytometer and analyzed with analysis software Kaluza; with BD FACSCantoIITM and analyzed with analysis software BDFACSDiva respectively. For each population analyzed with the two methods, the coefficient correlation (r) was calculated. Statistical analysys (correlation and Bland-Altman graphs) was performed through MedCal software. Results: The correlation result obtained between two methods of work was 99% on PB samples and 98% on BM samples. Concerning the monoclonal antibodies dispensing times, the two methods were significantly different: Solastra B method, 21.00", BD method, 90.18". Conclusions: The two methods are comparable as their correlation index is 99%. Solastra kit B enables the operator to reduce sample preparation time more than four times as well as enables the operator to reduce the probability of error in the volume of antibody released.

## IMMUNOLOGY

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NK CELL AND TUMOR MICROENVIRONMENT INTERACTIONS IN MELANOMA

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Although the NK cell capability of killing tumor targets in vitro has been well established; in vivo, and in particular, in solid tumors, their efficacy in clearing transformed cells has not yet definitely assessed. At the tumor site, different factors may indeed affect the activation status of NK cells and their migratory capabilities. By histological analysis we have evaluated the NK cell infiltrate in a series of melanoma lesions and we have assessed that: 1) the NK/Melanoma cell (NK/M) ratio in situ is far lower than that generally used *in vitro* to evaluate the NK cell killing capability; 2) in metastatic melanoma lesions NK cells are frequently associated to fibroblasts. By mean of co-culture experiments we have demonstrated how fibroblasts derived from metastatic melanoma lesions could severely interfere with the enhancement of the NK cell effector functions occurring during IL-2-induced activation, and how this phenomenon could profoundly impact on the ability of NK cells to kill tumor cells and to produce cytokines. In addition we also set up NK/Melanoma (NK/M) cells co-cultures at NK/M ratios similar those observed ex-vivo and observed that a part of melanoma cells survived and, more importantly, acquired resistance to NK cell-mediated killing. By the use of selective inhibitors and specific blocking mAbs we have also characterized the molecular bases of these two new mechanisms of tumor escape.

## ALTERATION OF Th17 AND TREG CELL SUBPOPULATIONS COEXIST IN PATIENTS AFFECTED WITH SYSTEMIC SCLEROSIS.

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Systemic sclerosis (SSc) is a chronic connective tissue disease characterized by vascular damage, autoimmunity, and excessive collagen deposition. Aim of the study has been to understand the relationship between Th17 and Treg cell subsets in patients affected with systemic sclerosis (SSc). Phenotypes and functions of pheripheral Th17 and Treg cell subsets were analyzed in a series of 36 SSc patients and 6 healthy controls by flow cytometry. Th17 cell frequency demonstrated a statistically significant increase in global CD4+T subset and particularly in CD4+CCR6+ T cells from SSc patients with respect to controls independently from type or stage of disease. Since CCR4 and CCR6 chemokine receptors identify a subset of TCD4+ producing IL-17 and specific for Candida albicans (Ca), the cytokine profile of TCD4+ repertoire has been analyzed after expansion with a polyclonal stimulus or Ca antigens. In both T cell lines the frequency of Th17 T cell clones resulted significantly higher in SSc patients with respect to controls suggesting the skewing of immune response in SSc patients toward Th17 cell generation/expansion. Concerning the Treg compartment, both CD4+CD25+ and CD8+CD28− Treg subsets were evaluated. When the CD4+CD25+highCD127low Treg was analyzed, decreased frequency and levels of suppressive activity were observed in patients with diffuse and active disease with respect to healthy controls. Regarding CD8+CD28-Treg, SSc patients showed increased levels independently from the type (limited or diffuse) of disease and alterated suppressor activity exerted by in vitro generated CD8+. Collectively, these data highlight the existence of an imbalanced ratio between Th17 and Treg cell subsets in SSc patients.

## IL-21 COUNTERACTS IL-2/TGF-β-MEDIATED INDUCTION OF REGULATORY T CELLS (Treg)

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Two major cues are involved in the increased propensity of T cells to become Treg and facilitates local tolerance restoration in the context of cancer immunotherapy: IL-2, which is administered to foster T cell proliferation and TGF-β, which is abundantly released by tumor cells and accumulates in tumor microenvironment. Immunomagnetically purified CellTrace Violet loaded $CD45RA^+$ naïve T cells were added back to the correspondent CFSE-loaded $CD45RO^+$ memory T cells, and activated by a concomitant engagement of CD2, CD3 and CD28 in the presence of IL-2 and TGF-β amounts apt to induce Foxp3 (300U/ml and 5ng/ml, respectively). Typically, IL-2/TGF-β combination induced Foxp3 expression in >25% naïve $CD4^+$T cells and ≤3% memory $CD4^+$T cells by day 5 of culture. IL-21 addition (100ng/ml) reduced Treg generation (≤7% $Foxp3^+$ cells among naïve $CD4^+$T cells). Mechanistically, IL-21 acted at two levels: it hampered Foxp3 expression at the early phases of T cell activation (72 hrs) in non dividing cells and favored non Treg over Treg proliferation in actively proliferating cells. Although IL-2 mainly phosphorylates Stat5, which is a Foxp3 positive regulator, Foxp3 downmodulation was not accompanied by changes in the phosphorylation status of this Stat. However, IL-21 promptly phosphorylated Stat3, which has an opposing role in Foxp3 regulation. Thus, it seems conceivable that IL-21-induced Stat3 phosphorylation prevailed over the downstream consequences of Stat5 phosphorylation. Finally, we show that contrary to the current belief, Treg suppressive activity on both $CD4^+$ and $CD8^+$ T cells resisted IL-21.

## CD63 EXPRESSION AS MARKER OF BASOPHIL ACTIVATION TEST

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In the last years the adverse drugs reactions have become more frequent, therefore new methods have assumed a role of support of clinic diagnosis. The aim of the study was to assess the basophil activation test by flow cytometry, reporting with the determination of specific IgE and with the clinical story of patients affected by adverse reactions to amoxicillin. So we used the FAST Immune kit (BD) by flow cytometry (FACSCantoII BD) and like allergens the amoxicillin and ciprofloxacin. This test bases on the expression of CD63, basophil activation marker, expressed after in vitro incubation with the allergens. We have studied 20 patients with documented adverse reaction to amoxicillin and 8 healthy volunteers, as control group with no history of adverse reaction to drugs. Peripheral blood samples were incubated with CD63FITC, CD123PE, HLA-DRPerCP MoAbs. 11/20 patients showed CD63 iperexpression, marker basophil activation. The specificity was 100%. 30% of the patients were positive in vitro to both antibiotics, although they reported adverse reaction only to amoxicillin. Besides, we described a strong and clear positivity for amoxicillin in a patient with anaphylactic shock after administration this antibiotic 5 months ago. Flow cytometry testing has a high specificity, so is able to identify all healthy donors, but it shows a low diagnostic sensitivity. However, within the optimal

time from an adverse clinical reaction, this test shows a critical role in allergy diagnostics.

## EVALUATION OF THE DEGRANULATION AND POLARIZATION PROCESSES INDUCED BY DIFFERENT NK ACTIVATORY AND COACTIVATORY RECEPTORS

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The exocytosis of perforins and granzymes contained into cytotolytic granules is the mechanism through which natural killer (NK) cells can spontaneously destroy tumour and pathogen-infected cells without a previous sensitization. The fine balance between signals coming from activatory and inhibitory receptors determines the granule polarization and the degranulation events, finally leading to target cell death.

To test the participation of different NK receptors in the polarization and degranulation processes, anti-Biotin MACSi-Beads loaded with combinations of biotinylated antibodies directed against surface activatory (NKp46) and coactivatory (2B4, DNAM-1, CD18 and CD2) molecules were co-incubated for 2 hours with peripheral blood lymphocytes and PerCp/Cy5.5-conjugated anti-CD107a monoclonal antibody (a marker of lymphocyte degranulation). NK cells were then stained with different antibodies (anti-CD16, -CD56, -KIRs and -NKG2A) and the degranulation of different NK cell subsets (CD56dim and CD56bright, licensed and unlicensed) were evaluated by flow cytometry. On the other hand, confocal microscopy allowed to evaluate the degree of granule polarization (a necessary step to induce target cell death) of these samples.

## INDUCIBLE EXPRESSION OF NEUROPILIN-1 (Nrp1) IN HUMAN T CELLS

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Literature data on Nrp1, a 130–140kDa cell surface glycoprotein initially described as involved in neuronal guidance and angiogenesis, in human T cells are bizarre. The original claim that Nrp1 is expressed by naïve T cells to facilitate the immunological synapse was later contradicted by the same Authors who also claimed Nrp1 not to be produced by T cells but passively acquired by trogocytosis. Subsequently, the same Authors showed that Nrp1 was produced by T cells following activation. Here we show that Nrp1 message and protein are induced in activated T cells. By western blot, the protein results of the same size (~140 kDa) as that expressed by differentiating monocytes and MDA tumor cells. A greatly suppressive $Nrp1^+$ regulatory T cell (Treg) subset characterized by a high CD25 expression colonizes tumor draining lymphnodes. Here we show that following activation, a fraction (~15%) of $CD4^+Nrp1^+$cells coexpresses $Foxp3^+$ and CD25, and the latter is markedly more expressed compared to corresponding $Nrp1^-$ cells. However, $Nrp1^+Foxp3^-$ cells also express a high level of CD25, suggesting that Nrp1 ultimately identifies strongly activated T cells, including Treg. A similar connection between Nrp1, Foxp3 and CD25 is observed also in activated $CD8^+$ cells, although $CD8^+Foxp3^+Nrp1^+$cells are virtually absent in tumor draining lymphnodes as well as in other lymphoid organs. This suggests that the modality of Nrp1 expression and perhaps its functional significance in $CD4^+$ and $CD8^+$ cells diverges. To conclusively address this issue, we are now exploring Nrp1 expression in $CD4^+$ and $CD8^+$ $Foxp3^+$ T cells generated in the presence of TGF-β and IL-2.

## MODULATION OF REGULATORY T CELLS IN MULTIPLE SCLEROSIS PATIENTS RESPONSIVE TO INTERFERON BETA THERAPY

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The response of multiple sclerosis to interferon-beta (IFNβ) therapy is highly variable, therefore biological markers predicting clinical benefits need to be identified. Since it is known that multiple sclerosis patients have a deficit of the regulatory T-cell (Treg) subsets, we investigated whether IFNβ induce differential modifications in the two main categories of Treg, the natural Treg and the IL-10-secreting inducible Tr1 subset. We studied 99 patients and, among them, 49 were treated with IFNβ for at least 24 months, while 50 began the treatment at the moment of the inclusion in the study and were then followed up for 12 months. Natural Treg cells were identified by flow cytometry as $CD4^+CD25^+CD127low/^-$T cells, while inducible Treg where studied indirectly by quantifying the expression of the RNA of IL-10 and CD46 by Real Time PCR after in vitro stimulation of lymphocytes with anti-CD46 and anti-CD3 monoclonal antibodies, which are known to expand a Tr1-like population.

IFNb induced a redistribution of the natural Treg, with a shift of the naive ($CD4^+CD25^+CD127low/^-CD45RA^+CCR7^+$) Treg subset towards the ($CD4^+CD25^+CD127low/^-CD45RA^-CCR7^+$) "central memory-like" Treg population.

Furthermore, in a subgroup of treated patients, the CD46/CD3 co-stimulation, probably through the Tr1-like subset modulation, increased the production of the RNA for IL-

10 and CD46. The same group showed a lower median Expanded Disability Status Scale after 2 years of therapy.

The selective increase of the "central memory-like" subset and the involvement of the Tr1-like population may be part of the mechanisms by which IFNβ achieves its beneficial effects. The quantification of the RNA for IL-10 and CD46 could be used to identify patient with different response to IFNβ.

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FOXP3 EXPRESSION ON $CD4^+$ $CD25^+$ LIMPHOCYTES FROM PATIENTS INFECTED WITH HIV, HCV OR HIV/HCV CO-INFECTED AND IN HEALTHY SUBJECTS: A PILOT STUDY

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Several studies have pointed out that HIV infection accelerates the progression of chronic C hepatitis. This is related to HCV virus replication and alteration of cell mediated immunity. Many lymphocyte sub-populations are involved in the modulation of immune system response and among them, $CD4^+CD25^+$ subset shows regulatory activity. A transcription factor (forkhead box P3, Foxp3) is correlated with the degree of liver fibrosis in HCV-coinfected subjects. In this paper we evaluate the levels of $CD4^+CD25^+$ with phenotype Foxp3 in $HIV^+$, $HCV^+$, $HIV^+/HCV^+$ pts versus a healthy control group. For each group, 20 pts has been considered. The determination and quantification of Treg cells have been carried out through a high resolution flow cytometric analysis with CD4, CD25 and Foxp3 MoAbs, each of them marked with a different fluorescence. The results show the following $CD4^+CD25^+Foxp3^+$ percentages: $HIV^+$ pts: range 0.70–5.73 %, median value 2.93%; $HIV^+/HCV^+$ pts: range 0.89–8.03%, median value 3.13%; $HCV^+$ pts: range 0.72–2.90%, median value 1.38%; control group: range 0.17–2.33%, median value 1.07%. Statistically significant differences ($p < 0.0005$, Kruskal-Wallis test and Dunn's Multiple Comparison test) were observed in $HIV^+$ and $HIV^+/HCV^+$ vs $HCV^+$ and control group. No statistically significant difference was observed in $HCV^+$ vs control group and $HIV^+$ vs $HIV^+/HCV^+$. Our preliminary results point out that the $HIV^+$ pts show levels of $CD4^+$ $CD25^+$ $Foxp3^+$ lymphocytes that are significantly higher than the healthy control and HCV groups. This activation of a regulatory subset lymphocyte may play a role in accelerating the course of liver disease. The dysregulation of Treg cells can inhibit the process of pathogens' elimination, making the disease evolve towards a chronic status.

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AIRE POLYMORPHISMS INDUCE VARIABLE EXTENT OF APOPTOSIS ON MELANOMASPECIFIC CD8+ T LYMPHOCYTES

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AIRE regulates thymocyte selection by inducing the expression of tissue-restricted self antigens (TRAs) in medullary thymic epithelial cells (mTECs). MAGE antigens are among TRAs regulated by AIRE. Single nucleotide polymorphisms (SNPs) of AIRE gene have been reported in humans but their impact on repertoire selection of tumor antigen-specific T lymphocytes is unknown. We report here that the rs1800522 SNP of human AIRE gene is present in mice and that the relative T or C allelic variants differently regulate MAGEB2 gene expression in mTECs. The C allelic variant, protective in humans against melanoma, induces lower MAGEB2 expression than the T allele. When mouse mTECs bearing the TT genotype were gene targeted to substitute the T with the C allelic variant, the co-culture of wild type or transgenic mTECs with MAGEB2-specific syngeneic T cells induced reduced extent of apoptosis and increased frequency of MAGEB2-specific viable T lymphocytes in T cells cultured with mTECs bearing the CC with respect to the TT genotype. These findings demonstrate that alternative allelic variants of AIRE SNPs may differently modulate the T cell repertoire specific against melanoma cells and subsequently may be linked to a different level of suceptibility to a neoplastic disease such as melanoma.

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CIRCULATING REGULATORY T-CELLS IN ''CLINICAL'' MONOCLONAL BCELL LYMPHOCYTOSIS

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Monoclonal B-cell lymphocytosis (MBL) is characterized by <5000/μL circulating clonal B-cells in absence of other features of lymphoproliferative disorders. Regulatory T-cells (Tregs) constitute a small subset of cells involved in antitumour immunity and are generally increased in patients with chronic lymphocytic leukemia (CLL), for the diagnosis of which >5000/μL circulating clonal B-cells are required. We evaluated the number of circulating Tregs, by means of multicolor flow cytometry (CD45/CD4/CD25/CD127), in 56 patients diagnosed with "clinical" MBL, 74 patients with CLL, and 40 healthy subjects, and compared it to clinical-biological features of the diseases. MBL patients showed a lower absolute number of Tregs compared to CLL patients ($p = 0.0004$), but higher than controls without statistical significance. Noteworthy, there was not a higher number of CD4+/μL cells within the CLL subset compared to MBL ($p = 0.16$). No significant correlation was found between Tregs number and CD38+B-cell/μL or ZAP-70. Moreover, the absolute cell number of Tregs

directly correlated both with more advanced Rai/Binet clinical stages ($p = 0.02$) and peripheral blood B-cell lymphocytosis ($p < 0.0001$). Of note, the absolute number of Tregs was found lower within MBL patients than within CLL patients staged as 0/A Rai/Binet ($p = 0.02$). This study showed that Treg cells are abnormally increased in "clinical" MBL patients, despite at a lower degree than in CLL patients. In light of these data, MBL seems to be a preliminar phase preceding the onset of CLL and the progressive increase of Tregs number might contribute to the evolution of MBL to overt CLL.

## IMMUNOMONITORING OF TRANSPLANTED PATIENTS INFUSED WITH MESENCHYMAL STROMAL CELLS (MSC) FOR TREATING STEROIDREFRACTORY GvHD

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The usage of MSCs as therapy for GvHD is constantly increasing, however clinical studies have been scarcely corroborated by biological analysis of patients response to cell infusion. We report the immunological monitoring of 10 patients with steroid-refractory acute or chronic GvHD, receiving multiple doses of MSCs. 2 patients responded completely (CR), 5 partially (PR) whereas 3 did not respond (NR) to MSCs. According to clinical observations, CR patients showed a significant decrease of three validated GvHD plasmatic markers, TNFRI, IL2Rα and elafin to the mean level of Healthy Donors (HD). In particular, at day +28, TNFRI decreased 2 times, IL2Rα decreased 1.9 times and elafin decreased 2.3 times. Moreover, investigating the effect of MSC infusions on lymphocytes circulating in the PB, in CR patients we observed a change in CD4+ T-cell subsets after therapy: Tregs increased, Th1 and Th17 populations decreased. In particular, Th1/Treg ratio decreased 3.5 times and Th17/Treg ratio decreased 3.6 times. Correspondingly, patient symptoms also gradually improved, suggesting an association between GvHD clinical course and CD4+ T-cell imbalance. In accordance with the decrease of Th1 CD4+ T cells in the PB of CR patients, we observed a valuable decrease of IFNγ plasma concentrations to the level typical of HD. Contrary to CR patients, in PR patients we observed a transient decrease of GVHD plasmatic markers and Th1/Treg, Th17/Treg ratios, while NR patients showed stable or even increasing levels of all analysed plasmatic and cellular markers.

In summary, despite its limited size, the present study suggests that MSCs, upon infusion, are able to convert an inflammatory environment to a more physiological one, both at a cellular and at a molecular level.

## IMMUNOBIOLOGICAL CHARACTERIZATION OF PRADERWILLI PATIENTS: QUALITATIVE AND QUANTITATIVE CYTOMETRIC STUDY

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The Prader-Willi Syndrome (PWS) is a genetic condition caused by an alteration of chromosome 15 (15q11-q13 marked portion). The disease is observed in only that people regard the lack of chromosome 15 of paternal origin.

The genes involved encode for one or more proteins presumably involved in brain development and its absence would lead to a generalized dysfunction of the brain, which affects the hypothalamic-pituitary region. The clinical features are characterized by neonatal hypotonia, dysmorphic notes, acromicria, hypogonadism, iposomia, obesity and behavioral problems. Neurobehavioral profile that includes cognitive deficits, learning disabilities, severe behavioral disorders. In this patients the saliva is thick and viscous and is associated with significant dental caries, oral hygiene is poor and the tooth enamel is usually poor and is easily eroded. This problem is increased by the grinding of the teeth and rumination. The diagnosis is still done with a delay even in the presence of clinical diagnostic criteria fairly reliable. It 'very important that the diagnosis is made early in order to obtain adequate prevention.

In the 25 patients enrolled in the study of this disease, the first test is the test we performed methylation was positive in 98% of cases.

Consequently, faced with a positive methylation test Prader Willi syndrome is necessary to make further inquiries such as the evaluation of the parameters salt, bacterial and cytological saliva, using flow cytometry we went to look for qualitative and quantitative change of lymphocyte subpopulations that are involved in recurrent infections. Experiments were conducted with specific antibodies for CD45, CD4, CD8, CD3 to evaluate the presence of subpopulation of lynfocyte.

## CATUMAXOMAB MEDIATES INCREASE OF ACTIVATED CYTOTOXIC CELLS IN ASCITES FLUID OF OVARIAN CANCER

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Catumaxomab (anti-EpCAM x anti-CD3), a trifunctional monoclonal antibody (MoAb), binds to $EpCAM^+$ tumor cells and $CD3^+$ T cells, and simultaneously recruits NK cells, macrophages and dendritic cells via binding of its Fc-region to Fcγ-receptors type I, IIa and III. Malignant ascites can be treated with intraperitoneal catumaxomab infusions, as EpCAM is expressed on most epithelial cancer cells but not on mesothelial or blood cells. We analyzed immune cells in the ascites fluid of 6 ovarian cancer patients from study IP-CAT-AC-03/CASIMAS prior to first (baseline) and prior to the fourth catumaxomab infusion (10 days later). Half of the patients received prednisolone premedication (25mg) to attenuate cytokine release ensuing catumaxomab administration. As catumaxomab may interfere with binding of other MoAb to CD3, total T cells were identified as $CD2^+CD56^-$cells while NK and cytotoxic T cells were collectively identified as $CD2^+CD56^+$cells. At baseline, T/NK cells and phagocytes (HLA-$DR^+$ and/or $CD14^+$ cells) among viable mononuclear leukocytes were ∼50% and ∼35%, respectively. Cytotoxic cells were ∼15% among T/NK cells. Following catumaxomab infusion, the T/NK cell proportion increased to ∼75% and cytotoxic cell proportion to ∼25%. Phagocytes were ∼10%. Activation marker expression (CD69 and CD38) significantly increased in T/NK cells, but not significantly in phagocytes. Exfoliated tumor cells, assessed by a non-competing anti-EpCAM MoAb were detected in most patients at baseline and never thereafter. These findings suggest that catumaxomab eliminates tumor cells via activation of local immune response mechanisms. Interestingly, prednisolone premedication did not affect catumaxomab mediated immune cell activation.

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SEVERE DEPLETION OF CIRCULATING DENDRITIC CELL SUBSETS IN HODGKIN DISEASE: PRELIMINARY RESULTS

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Two main circulating dendritic cell (DC) subsets, namely myeloid (m) and plasmocytoid (p) DCs, have been described in humans. Myeloid DCs comprise type-1 mDCs, that are the main sentinels driving cell-mediated immune responses, and the poorly characterized type-2 mDCs. Plasmocytoid DCs are thought to be involved in immune tolerance and exert potent anti-viral and antitumour activities. Hodgkin's disease (HD) is characterized by only a few malignant cells and an abundance of inflammatory and immune cells representing a functional part of the disease. As to our knowledge no study has so far analyzed the different DC subsets in HD, the phenotype and frequency of blood DC subsets were assessed by multi-parametric flow cytometry in 16 HD patients at the time of diagnosis (M:F = 6:10, mean age 39.1 yrs) and 15 age and sex matched healthy volunteers. The study population was enrolled at the Haematology Oncology Division of the National Cancer Institute "Pascale Foundation" of Naples while control subjects were recruit- XXIX Conferenza Nazionale di Citometria Centro Congressi Grand Hotel ed among hospital staff. The proportion and absolute number of type-1 mDC, type-2 mDC and pDC were all significantly reduced in HD patients as compared to the control group ($p < 0.001$ in all instances). These findings were associated with a marked increase of the mDC/pDC ratio in HD cases ($29.9 \pm 53$ *vs* $1.6 \pm 0.6$) which was however not statistically significant due to the wide range of recorded values. Interestingly, a slight correlation of the proportion of pDCs with that of $CD4^+$ T lymphocytes was observed in HD patients ($p = 0.047$; $r = 0.54$). This is the first preliminary report of a severe depletion of circulating DC subsets in HD. Further efforts to assess the pathogenetic and clinical relevance (disease severity and therapy response) of these findings are needed.

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EVOLUTION OF LABORATORY PARAMETERS IN A PRETERM WITH INFLUENZA A H1N1 TRANSMISSION FROM MOTHER TO FETUS

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Introduction The influenza A H1N1 is an acute viral respiratory infection with symptoms similar to those with classical influence. In literature there have been no cases of vertical transmission of this influence.

Scope of Work Describe the evolution of hematological parameters and laboratory in a case of influenza A H1N1 infection from vertical transmission.

Material and methods The baby is preterm, 31 wks, kg. 1820, by emergency caesarean (TC) from mother hospitalized in intensive care for respiratory complications from influenza A H1N1. Aseptic standards were used according to the CDC in course of H1N1 infection. The baby was intubated at birth, placed on mechanical ventilation and promptly placed in isolation. The blood count showed 5.82 GB (×10 ^uL) with 18.3% neutrophils, platelet count normal, CRP 0.28 mg/dl blood culture negative, while the Chest X-ray was suggestive of hyaline membrane disease lung. In the third day, after a transient improvement of clinical condition, the child has shown a sudden deterioration with signs of severe respiratory distress due to viral pneumonia influenzaAH1N1 and had GB 14.33 (×10 ^uL), neutrophils 25.4% ratio, I/T 0.23 and CRP 21.5 mg/dl.

Conclusion The implementation of the buffer on the health workforce that has had any contact during the transport of newborn in UTIN has absolutely ruled out any possibility of exogenous contamination. The close collaboration with the laboratory made it possible to track the progress of hematological, virological and laboratory correlation with the clinical course of disease. The child was followed in follow-up and did not complication to distance.

## HUMAN DECIDUA CONTAINS NK CELL COMMITTED CD34$^+$ HEMATOPOIETIC PRECURSORS ABLE TO DIFFERENTIATE INTO FUNCTIONAL NK CELLS

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CD56bright decidual Natural Killer (NK) cells display a unique functional profile and play a key role in promoting tissue remodeling, neo-angiogenesis and immune modulation aimed to promote fetal tolerance. Little information exists on their origin and development. Here we show that human decidua contains CD34$^+$VEGFR$^-$hematopoietic cell precursors (dCD34$^+$). Comparative flow cytometric analyses between peripheral blood, cord blood and decidual CD34$^+$ cells showed that dCD34$^+$ cells express CD122 and CD127. Surface expression of these molecules, together with the presence of mRNA for E4BP4 and ID2 transcription factors, suggests that dCD34+ cells are committed to the NK cell lineage. dCD34$^+$ precursors undergo in vitro differentiation into functional CD56 bright NCR$^+$CD16$^-$KIR$^{+/-}$ NK cells in the presence of growth factors and are able to secrete IL-8 and IL-22. Moreover co-culture of dCD34$^+$ cells with decidual stromal cells, in the absence of exogenous cytokines, is sufficient to induce NK cell differentiation. Our findings strongly suggest that decidual NK cells may directly derive from CD34$^+$ cell precursors present in the decidua upon specific interactions with decidual microenvironment.

## SOLUBLE HLA-G DAMPENS CD94/NKG2A EXPRESSION AND FUNCTION AND DIFFERENTIALLY MODULATES CHEMOTAXIS AND CYTOKINE/CHEMOKINE SECRETION IN CD56$^{bright}$ AND CD56$^{dim}$ NK CELLS

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Soluble HLA-G (sHLA-G) inhibits different functions of natural killer (NK) cells. Here we investigated sHLA-G-mediated modulation of i) chemokine receptor expression and chemotaxis, ii) NK cell receptor expression and function and iii) secretion of cytokines and chemokines in CD56bright and CD56dim NK cells.

Peripheral blood (PB) and tonsil NK cells were cultured in the presence or absence of sHLA-G. Next, chemokine receptor and NK receptor expression was analyzed by flow cytometry. sHLA-G downregulated i) CXCR3 on PB and tonsil CD56$^{bright}$ and CD56$^{dim}$, ii) CCR2 on PB and tonsil CD56$^{bright}$, iii) CX3CR1 on PB CD56$^{dim}$, iv) CXCR5 on tonsil CD56$^{dim}$ and v) CD94/NKG2A on PB and tonsil CD56$^{bright}$ and CD56$^{dim}$ NK cells. Addition of anti-HLA-G or anti-ILT2 mAbs significantly restored CXCR3 and NKG2A expression, thus indicating that downmodulation of these molecules was mediated by sHLA-G, mainly through ILT2 receptor.

Chemotaxis of i) PB NK cells towards CXCL10, CXCL11 and CX3CL1 and ii) CD56bright PB NK cells towards CCL2 and CXCL10 was inhibited by sHLA-G. Interferon-□ secretion induced by NKp46 engagement in PB NK cells was inhibited by NKG2A engagement in untreated but not in sHLA-G-treated NK cells, thus indicating that CD94/NKG2A inhibitory function was dampened by sHLA-G. sHLA-G augmented the secretion of i) CCL22 in CD56$^{bright}$ and CD56dim and ii) CCL2, CCL8 and CXCL2-CXCL3 in CD56$^{dim}$ PB NK cells. Signal transduction experiments showed that Stat5 but not Stat1 and XXIX Conferenza Nazionale diStat3 phosphorylation was downmodulated by sHLA-G in PB NK cells.

In conclusion, we delineated novel mechanisms of sHLAG-mediated inhibition of NK cells, with differential inhibitory effects on CD56$^{bright}$ and CD56$^{dim}$ NK cells. Finally, we described a novel sHLA-G-mediated negative feedback on CD94/NKG2A receptor.

## FUNCTIONAL ADCC EVALUATION IN METASTATIC COLON CANCER (mCRC) PATIENTS IN TREATMENT WITH CETUXIMAB: THE ROLE OF FCGAMMA II AND FCGAMMA3 POLYMORPHISM

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Activity of cetuximab, a chimeric monoclonal antibody targeting the epidermal growth factor receptor, is largely attributed to its direct antiproliferative and proapoptotic effects. Previous studies demonstrated a correlation between the predictive response to cetuximab and the expression of specific Fcgamma IIA and IIIA polymorphism correlated to an ADCC activity.

The Fcgamma II (HH vs HR vs RR) and Fcgamma III polymorphism (VV vs VF vs FF) was evaluated in 49 patients affected with mCRC in treatment with cetuximab and correlated to the clinical response. Out of 49 patients evaluated 18 Fcgamma II A H/H and 27 H/R, considered to be better responder, showed 3 RC, 14 RP, 11 SD e 17 PD while 4 RR (worse responder) gave 0 RC, 0 RP, 3 SD and 1 PD. The polymorphism Fc gamma IIIA 21 V/V and 22 V/F (better responder) showed 2 RC, 16 RP, 12 SD and 12 PD while 6 F/F (worse responder) showed 0 RC, 4 RP, 0SD and 2 PD. CD107a (LAMP-1), a marker for degranulation of NK (CD16+/CD56+) and activated CD8+ T cells was evaluated for ADCC activity in patients treated with cetuximab in the presence of human colon cancer cells (HT29) EGF-R overex-

pressing and cetuximab. Preliminary results showed correlation between ADCC activity and cetuximab responsiveness.

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IMMUNOPHENOTYPICAL AND FUNCTIONAL CHARACTERIZATION OF FIBROBLASTS IN SSc PATIENTS

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Systemic sclerosis (SSc) is a clinically heterogeneous, systemic disorder which affects the connective tissue of the skin, internal organs and the walls of blood vessels. Fibroblasts are essential in the pathogenesis of SSc and the resultant skin thickening and interstitial lung fibrosis. Dermal fibroblasts were isolated from lesional or not skin of SSc patients at different disease stages [diffuse (D)/limitated (L) and active (A)/non active (NA) form] and healthy donors and cultured in vitro. All fibroblasts were characterized immunophenotypically (CD14-Citometria Centro Congressi Grand Hotel Salerno 58 ottobre 2011 Salerno, CD34-, HLA-DR-, CD31-, HLA-I+, CD44+, CD105+, CD106+, CD29+, CD166+, CD73+, CD49d+) and evaluated for several functional properties by cytometric approaches. The analysis of proliferation potential by dye dilution assay showed that fibroblasts derived from SSc patients (D and/or A form) exhibit decreased proliferating precursor frequency. We tested also the type I collagen by ELISA, since excess deposition and accumulation of extracellular matrix is the the hallmark of SSc disease. Fibroblasts from SSc patients resulted functionally activated showing an increased basal production of collagen I. Moreover, we evaluated IL-6 production in medium daily for a week. We found that IL-6, a pro-inflammatory cytokine that would appear to participate in the excessive of SSc fibroblasts, is significantly higher in DAvs healthy donors, LNA and DNA patients. We co-cultured fibroblasts of different patients with CD4+ of healthy donor for five days: % of CD4+CCR6+ cells does not change compared to CD4+ ex vivo, but increase % of cells CCR6+ producing IL17. Finally, fibroblasts of A/D stages are functionally different by healthy and L/NA, and capable of modulate T-response.

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BASOPHILS AUTOINDUCED DEGRANULATION (BAD) STIMULATED WITH AUTOLOGOUS SERUM, BY A TWO COLOURS METHOD FLOW CYTOMETRY. A SUITABLE SUPPORT FOR THE DIAGNOSIS OF CHRONIC AUTOIMMUNE URTICARIA

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Urticaria is a syndrome that includes a wide range of diseases. Since 1962 Rorsman hypothesized that a proportion of chronic idiopathic urticaria is the expression of an autoimmune process dependent by IgG and, sometimes, IgE, subsequently confirmed by Hide and Niimi. Later the involvement of the complement in this process was demonstrated too.There aren't clinical or histological features that can be used as a paradigm in the diagnosis of CAU. The foundation of the diagnosis is the demonstration of the presence of anti-FcεRIα and anti-IgE autoantibodies in the serum of patients. The Autologous Serum Test (ASST) is an useful screening procedure, but the sensitivity and specificity is of 70–80% and this test requires further confirmatory methods. BAD test, marking CD63, performed on whole blood stimulated with autologous test, is useful for the quantitative determination of the basophils degranulation by flow cytometry. Recent studies have shown that the sensitivity and specifity of this method is of 95,5% and 90,5% respectively. With heparinized blood of patients with CU we prepared negative controls, positive controls and the samples stimulated with autologous whole and decomplemented serum., and everything was labeled with mo-Ab anti-CD63-FITC and anti-CCR3-PE, incubated, lysed and read by flow cytometry. BAD test, performed on 100 patients with CU, have evidenced that 24 of these showed an increase of CD63 on basophils stimulated with whole and/or decomplemented serum and hence they suffered from CAU. Our results lead us to hope that BAD could become a routine test in the diagnosis of CAU., to perform before ASST. The sensitivity of BAD could be enhanced by associating the basophil activation marker CD203c.

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SUGGESTIONS FOR A PROPER DIAGNOSIS OF HYPERSENSITIVITY TO DRUGS AND EXCIPIENTS BY FLOW CYTOMETRY

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Identification of the antigens responsible for allergic reactions is essential both for diagnostic purposes and for an effective prevention. Aim of our work was to demonstrate that a complete diagnosis of hypersensitivity to such drugs should also include the analysis of the excipients. In this case the attention was focused on hypersensitivity reactions to macrolides and especially to Clarithromycin and to understand if hypersensitivity reactions had to the active substance or to dye E104 (quinoline yellow) this in commercial sizes of medicine. Basophil Activation Tests (BAT) 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 with in IRMA laboratories, together with a sample of 30 subjects with suspected hypersensitivity to clarithromycin, we carried out the tests on 5 atopic subjects, sensitized to various allergens but taking clarithromycin hadn't allergic reaction, in order to confirm the specificity of test. BATs revealed a prevalence of positivity for excipient, in fact 10 patients (33.34 %) showed positive reaction to the stimulus with clarithromycin, 16 (53.34 % ) with quinoline yellow and 6 subjects (20%) showed positive to both the molecules. This study shows the importance of run BAT for E104 on all patients suspected of Clarithromicin sensitization as this dye is contained in the most common commercial form.

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## ''UNCONVENTIONAL'' LYMPHOCYTES: GAMMADELTA T CELL EXPANSION IN PERIPHERAL BLOOD SAMPLES

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T lymphocytes bearing the gamma-delta T cell receptor (TCR) represent a minor subset among peripheral blood lymphocytes (1–5%); the majority of them do not express CD4 and CD8 molecule.

As component of the innate immune population, gamma-delta T cell are involved in immunosurveillance and host defense against intracellular pathogens but also show an immunoregolatory function, mainly through cell-to-cell contact. The hightly limited T cell receptor V region repertoire is one of the most important feature distinguishing these lymphocytes from MHC-restricted alfa-beta T cells; a great cytotoxic potential and the ability to produce proinflammatory cytokines upon activation are other their hallmarks. Vgamma9Vdelta2 T cells, the major subset of the human adult peripheral blood, ranging from 80–90% of the gamma-delta T pool, specifically recognize non peptidic phosphomonoester sequences, also present in mycobacterial cell wall, proliferate and seem to develop some feature of memory cells.Several studies have demonstrated a gamma-delta T cell expansion in mycobacterial infection.

An expanded gamma-delta T population (13% and 40% of T lymphocytes) in a peripheral blood sample of two patients ospedalized in San Giovanni,Roma,is reported in this note. Interestingly this population displayed an effector memory phenotype,with a prevalence of CD45RA+ CD62L-CD16+ "terminally differentiated" T effector memory cells (TEMRA).- This phenotype has been interpreted as "reactive" in subjects who experienced Mycobacterium tuberculosis infection.

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## DIFFERENTIAL ACTION OF 3HAA ON VIABILITY AND ACTIVATION OF STIMULATED LYMPHOCYTES

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Antigen driven lymphocyte activation and proliferation are necessary for the development of adaptive immunity, both in physiologic and in pathologic conditions. This mechanism leads to an increase in lymphocyte count, which usually lasts some weeks until activation-induced cell apoptosis allows the homeostatic control of the system. Indeed, in the first days after stimulation, activated lymphocytes display high resistance to apoptosis and to most immunosuppressive drugs. However, according to the literature, few compounds have been described to kill recently activated cells, by inhibiting metabolic processes fundamental to proliferation. The aim of our work was to evaluate comparatively these different compounds, in order to identify the best strategy to kill cells that have undergone proliferation, while sparing the repertoire of resting cells.

After preliminary experiments, 3-HAA and bortezomib were selected as the most suitable drugs for our purposes. The possible synergic effect of 3-HAA with bortezomib or with manganese ions was also assessed.

3-HAA was confirmed to be the most reliable pharmacologic approach to inhibit proliferation with acceptable toxicity on resting cells. Manganese ions can further enhance this effect, while results with bortezomib seem to be less consistent.. While in the case of PHA simulation 3-HAA lead to death of most lymphocytes, only a minor percentage of cells were killed after allo-stimulation, suggesting that the effect is proportional to the percentage of stimulated lymphocytes.

These results deserve further investigations to develop new procedures for targeting of activated cells with pharmacological approaches.

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## THE ROLE OF CXCR7 IN HUMAN RENAL CANCER CELLS

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The alternative CXCL12 receptor, CXCR7, was recently described in a variety of human cancers. Nevertheless its function and transduction pathway is not clarified yet. In this study CXCR7 expression was evaluated in human renal cancer cell lines with different CXCR4 and CXCR7 expression level (RXF393 high levels of CXCR7 compared to CXCR4, A498 high expression of both receptors and SN12C, in which the two receptors are poorly represented). To dissect

the CXCR4 and CXCR7 pathway siRNA for CXCR4 and CXCR7 were transduced in SN12C and RXF393 cells. In SN12C siCXCR4, P-Erk is induced by CXCL12 but is not inhibited by AMD3100 nor by AbCXCR4 while AbCXCR7 inhibited the induction. In SN12C siCXCR7 PErk is induced by CXCL12, is inhibited by AMD3100 and by AbCXCR4 while AbCXCR7 did not inhibit the CXCL12 induction. Likewise in the RXF393 siCXCR4 or CXCR7. CXCR7 is an active signalling receptor in RCC cell lines suggesting an accessory role for CXCR7 with a central role in cell growth. Thus the concomitant inhibition of CXCR4 and CXCR7 pathway may be effective alone or in combination with anti-angiogenic drugs in the therapy of renal cell carcinoma.

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MELANOMA CELLS INTERFERE WITH THE ACTIVITY OF NK CELLS BY MODULATING THEIR PHENOTYPE AND CYTOTOXIC FUNCTION

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Natural killer cells are thought to play a relevant role in the immune surveillance against tumors and the degree of NK cell infiltration at the tumor site has been shown to directly correlate with a better prognosis. Adoptive immunotherapy protocols using NK cells had limited clinical efficacy, possibly related to tumor escape mechanisms causing inhibition of NK cell function. This may reflect the production, by tumor cells, of cytokines and/or other inhibitory factors. To date, limited information is available on the effect of melanoma cells on NK cell activity. In this study we analyzed the consequence of melanoma/NK cell interactions on NK cell phenotype and function. We show that melanoma cells can inhibit the expression of major surface triggering NK receptors including NKp30, NKp44 and NKG2D with consequent impairment of NK cell-mediated cytolytic activity against various melanoma cell lines. This inhibitory effect was primarily mediated by indoelamine 2,3-dioxygenase (IDO) and prostaglandin E2 (PGE2). These data suggest that tumor-derived immunosuppressive factors may greatly hamper the anti-tumor activity of human NK cells, thus favouring tumor progression.

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ALLERGEN-INDUCED BRADYKININ B2R EXPRESSION IN MILD ATOPIC ASTHMA

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Chronic asthma is an inflammatory disease with different degrees of airway remodeling, involving various cell types including fibroblasts and myofibroblasts. Bradykinin (BK) mediates bronchial inflammation and damage in the early phase of acute allergic asthma, and may also play a major role in airway remodeling in chronic asthma. We studied the expression of BK B2 receptor (B2R) in bronchial biopsies and in primary fibroblasts from asthmatics.

B2R expression was examined by immunohistochemistry and localized by confocal microscopy in bronchial biopsies from 10 asthmatics after diluent and allergen challenges. In lines of bronchial fibroblasts from 3 asthmatics (HBAFb) and in 2 other lines of control fibroblasts we evaluated B2R expression, by western blotting (WB) and aSMA expression by immunocytochemistry or WB.

Asthmatics had higher $B2R^+$ in the submucosa compared to controls. The number of $B2R^+$ cells in biopsies was increased after allergen challenge in asthmatics, and this increased expression was localized in fibroblasts and myofibroblasts of the bronchial submucosa. *In* vitro, B2R was over-expressed in asthmatic bronchial fibroblasts as compared to control cells. Furthermore, BK induced fibroblast proliferation and a-SMA expression via B2R.

These data suggest a novel and central orchestrating role of BK in fibroblastdriven airway remodeling, a process that underlies the development of fixed airway obstruction in asthma.

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APOPTOSIS OF MBPSPECIFIC LYMPHOCYTES IS MODULATED BY TIM-3 IN PATIENTS WITH MULTIPLE SCLEROSIS

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T cell immunoglobulin domain and mucin domain-containing molecule-3 (Tim-3) is expressed on a variety of immune cells and is involved in the regulation of innate and adaptive immune responses. In the initial phase of the immune response, DC-expressed Tim-3 is suggested to promote inflammation by synergizing with TLRs. Once an effector response is generated, Tim-3 is expressed by terminally-differentiated Th1 cells; these cells up-regulate Galectin-9 expression through the production of IFNγ. The interaction between Galectin-9 and Tim-3 results in the inhibition of Th-1 responses and cell death. T-cell clones derived from the CSF of Multiple Sclerosis (MS) patients produce higher levels of IFN-γ and express lower levels of Tim-3 compared to healthy control (HC); resident microglia and infiltrating monocytes, cells that contribuite to the MS-associated CNS inflammation, also expressTim-3. We analysed by flow-cytometry Tim-3 expression on MBP-stimulated CD4+ and CD8+ T lymphocytes and CD14+ cells, as well as Annexin V expression and IFN-γ production in 50 MS patients with a diagnosis of relapsing-remitting (RR), primary progressive (PP), secondary progressive (SP), or benign MS (BMS). Forty age and sex

matched healthy controls (HC) were also enrolled in study. Results showed that in BMS patients MBP-stimulated: 1) Tim-3 –expressing CD4+ and CD8+ T-cells; 2) CD4+AV+Tim-3+ and CD8+ AV+ Tim3+ (apoptotic cells); were significantly augmented compared to the values observed in the other groups of patients; results observed in HC were comparable to those seen in BMS patients. These data suggest that the maintenance of a physiologic Tim-3 expression is associated with a benign course of MS, possibly via the induction of apoptosis of MBP-specific lymphocytes.

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LANCL2 IS THE ABSCISIC ACID RECEPTOR IN HUMAN GRANULOCYTES AND IN RAT INSULINOMA CELLS

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Abscisic acid (ABA) is a plant hormone regulating fundamental physiological functions in plants, such as response to abiotic stress. Recently, ABA was shown to be produced and released by human granulocytes, by insulin-producing rat insulinoma cells and by human and murine pancreatic beta cells. ABA autocrinally stimulates the functional activities specific for each cell type through a receptor-operated signal transduction pathway, sequentially involving a pertussis toxin (PTX)-sensitive receptor/G-protein complex, cyclic AMP, CD38-produced cyclic ADP-ribose and intracellular calcium. Here, the ABA receptor on human granulocytes and on rat insulinoma cells is identified as the lanthionine synthetase C-like protein LANCL2. Co-expression of LANCL2 and CD38 in the human HeLa cell line reproduces the ABA-signaling pathway. The PTX-sensitive G protein coupled to LANCL2 is identified as Gi by transfection of $CD38^+$/$LANCL2^+$ HeLa with a chimeric G protein (Gαq/i). Identification of the mammalian ABA receptor will enable the screening of synthetic ABA antagonists as prospective new anti-inflammatory and antidiabetic agents.

INFLUENZA VACCINE ENHANCES IL-22 AND IL-17A PRODUCTION POTENTIATING Vγδ1 T CELL REACTIVITY TO CANDIDA ALBICANS IN HIV-$1^+$ ADULTS

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We reported that in HIV-1 infected patients circulating Vdelta1 T lymphocytes (Vδ1) increase and proliferate in vitro in response to Candida albicans (Ca). Herein, we analysed the effects of influenza vaccination, with vaccine containing or not the MF59 adjuvant, on the Vdelta1 T cell responses to hemagglutinin (HA) and Ca in HIV-1 seropositive and seronegative adults, to clarify the molecular mechanisms triggered in vivo by an adjuvanted vaccine against influenza virus. Seropositive ($HIV\text{-}1^+$) and seronegative ($HIV\text{-}1^-$) subjects received vaccines containing or not the MF59 adjuvant and the effects on T cell proliferation or cytokine production (IL-17A, IL-22, IL-23, IL-6) to antigens were evaluated at t0 and day 30 and 90 after vaccination by cytofluorimetric approaches. The data showed that: i) a population of circulating Vδ1T lymphocyte is expanded in vivo in HIV-1 seropositive subjects; ii) this population is capable of proliferating and enhancing cytokine production, including IL-17A and IL-22, in response to Ca, and IL-23 or IL-6 in response to HA; iii) these responses are enhanced upon influenza virus vaccination with a vaccine containing the MF59 adjuvant.

We suggest that in HIV-1 infected patients the circulating Vδ1 T lymphocytes reactive to Ca upon challenge with influenza virus vaccine receive an activating/enhancing signal mediated by cytokines triggered by the boost with HA antigen particularly in presence of MF59 adjuvant Thus the expansion of a specific circulating γδ T cell population, capable of producing anti-fungal cytokines, enhanced by influenza vaccine administration, in HIV-1 infected subjects might participate in to the control of HIV-1 spreading and to the defence against opportunistic infections, possibly contributing to compensate the impairment of $CD4^+$ T cells.

## METHODOLOGY AND TECHNOLOGY

GMP QUALITY CONTROL FOR ADVANCED THERAPY MEDICINAL PRODUCTS (ATMPs): FLOW CYTOMETRY VALIDATION

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Recent insights from regulatory Authorities request GMP quality standards for production and quality controls for ATMPs. Cell factories need to validate their production

process following GMP requirements and they have to guarantee appropriate quality control points all along their path prior to batch release. To this end we have implemented a throughput quality control system to support ATMPs in process and batch release. Here the FACS GMP validation process is described.

The FACS validation process was conducted according to international guidelines (ICHQ2-R1) and European pharmacopoeia. The procedure has been divided in two phases. The first phase was dedicated to FACSCanto II® setting and instrument performance qualification by using CST® (BD) and Rainbow® (Spherotech) beads. The second phase was the validation of the 7 colour Immunophenotyping analysis of human MSCs to assess specificity and repeatability. mAbs have been chosen based on literature data. Below minimal criteria to define human MSCs are reported: they are CD73, CD105, CD44, CD90 and CD29 positive and CD45, CD14, CD19 and CD34 negative. Specificity was focused on showing that mAbs used were able to identify unequivocally targeted antigens, to this aim the approach used was the Fluorescence Minus One (FMO) controls.

Repeatability was assessed performing three independent experiments.

The analysis of FACSCanto II® PMT voltage values showed steady and highly reproducible instrument performance. The Immunophenotyping validation showed that each FMO control was comparable to the respective negative control. Results obtained are in line to literature data and suggest high specificity and repeatability of the method. In order to support our validation we used the Immunophenotyping panel to qualify human MSCs cell lines obtained by different.

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## COMPARISON OF COMPUTATIONAL METHODS FOR THE ESTIMATION OF THE DIELECTROPHORETIC FORCE ACTING ON BIOLOGICAL CELLS AND AGGREGATES IN SILICON LABONCHIP

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Dielectrophoresis (DEP) has been reported as a promising method for cell manipulation without physical contact in miniaturized devices since it exploits the dielectric properties of cells suspended in a fluid and subjected to a variable gradient electric field E.

The mathematical expression of the DEP force is obtained by a multipole expansion involving increasing power of the particle's radius r and subsequent field derivatives. When the aggregate's radius and/or the field non-uniformity factor increase, more terms of higher order electric field derivatives in the multipole expansion should be considered. In this way the accuracy is improved but the numerical stability is worsened due to the high order derivatives operator. For this reason, a discrete method for the force computation is used: cell aggregates are virtually divided into small enough regions, the dipole DEP force value is computed on each of them and all the contributions are summed up to get the overall acting force. This method results to be more stable but also computationally more expensive.

The aim of this work is to find two numerical indices that permit to decide, given the aggregate radius and the field non-uniformity estimate, which computational method should be used: dipole, quadrupole or discrete approximation. Some functions for the measure of the difference between the various methods' solutions are defined and suitable threshold values are chosen allowing the creation of a graph that, given the aggregate's radius and the field non-uniformity estimate, shows which method should be used.

The comparison with some experiments is performed both in case of single Saccharomyces cerevisiae yeast cells and of Langerhans islets cellular aggregates and the results agree with the simulations.

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## IRIS™Dots NANOPARTICLES AS A NOVEL TOOL FOR FLOW CYTOMETRY ANALYSIS AND MULTI CELLULAR CULTURE APPROACH

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Nanotechnology is currently undergoing development in biological field and plays an innovative role in drug delivery, imaging and biosensors. New approaches using nanoparticles (NPs) such as quantum dots and iron oxide

NPs have been reported in several papers about cell imaging and cytometry. QDot™ were the first fluorescent nanoparticles used in cytometry, since they are very bright, photostable, insensitive to environmental factors and they can be excited in broad visible spectra. There are a great number of publications about their use in vitro applications, however there are contrasting views about their cellular toxicity. Different manufacturing strategies were used to avoid cell damage, e.g. the use of biocompatible shell, such as silica. IRIS™ Dots are silica NPs where indocyanines IRIS™ (Cyanine Technologies Spa, Torino) dye are covalently bound to silica matrix, resulting in an increase of the quantum yield of the fluorophores. IRIS™ Dots can be internalized in cells, by 30 minutes incubation in serum-free isotonic medium (NaCl 0.9%). IRIS™Dots were tested in classical antiproliferative assay, showing no acute toxicity after 72h in A2780, HCT116, HT1080, and MCF-7 cell lines. Despite sub-optimal excitation, stained cells and analyzed by BD FACSAria™ cytometer were clearly separated respect to control cells. IRIS™ Dots 2, 3 and 5 allowed the effective staining of several cell lines, showing possibility to use this approach for multi cellular culture, where is possible to analyze cellular populations without antibodies or other cell type specific reagents.

## A CELL-BASED OPTICAL BIOSENSOR (SILICON PHOTONIC CRYSTAL): A NEW TOOL FOR MONITORING CELLULAR ACTIVITIES

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The research investigates the use of silicon micromachined photonic crystals (PC) applied as micro-optodevices for in vitro monitoring of cellular activities (proliferation, differentiation and/or apoptosis/death). The proposed microsystem realizes a sort of silicon microincubator for cell cultures in a three-dimensional system in which the core element is a silicon photonic crystal. PCs are artificial materials characterized by the presence of photonic band gaps, corresponding to wavelength intervals where the propagation of the electromagnetic field inside the material is prohibited. We plan to use vertical, high aspect-ratio silicon photonic crystal, formed by a periodic array of parallel silicon walls separated by air gaps fabricated by electro-chemical etching of <100> oriented silicon. In this project, PC reflectors, are formed by periodic arrays of ≈3 μm thick silicon walls separated by ≈5 μm wide gaps. The presence of the cells, expected to extend their body or part of it inside the gaps, affects the optical properties of the device, strongly dependent on the refractive index of the gap filling material, thus changing the spectral position of the band gaps. After the initial cell culture inside the microincubator monitored by a variety of cytometric techniques (from conventional bright field and fluorescence microscopy to confocal microscopy supported by flow cytometry before and after culture), spectral reflectivity measurements are performed in the wavelength range 1.0–1.7 μm where biological materials do not show interfering effects such as absorption and fluorescence. We plan to use, as biological model the transition from epithelial to mesenchymal cells (EMT), investigating the morphological changes induced by the transition. Demonstration of the functionality of this device incorporating the cell-model, combined with an intrinsic optical monitoring method based on spectral reflectivity measurements using a fiberoptic setup, represents the initial step toward the development of a label-free cell-based biosensor, capable of responding to a wide range of biologically active compounds. Applications of our results can be in the field of drug discovery and, more in general, for the elaboration of innovative diagnostic and therapeutic strategies.

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## BCR-ABL ONCOPROTEIN DETECTION IN CEREBROSPINAL FLUID *VIA* FLOW-CYTOMETRY IMMUNOBEAD ASSAY

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Central nervous system (CNS) is a well-known sanctuary site for B-origin acute lymphoblastic leukemia (B-ALL) and chronic myeloid leukemia in lymphoid blast crisis (ly-BC-CML). Factors predicting a CNS involvement increased risk include the presence of leukemic cells in cerebrospinal fluid (CSF) and a Philadelphia (Ph)-positive genotype. Despite the introduction of effective CNS prophylaxis programs in the context of imatinib-based therapy schemes decreased CNS disease risk, a restricted percent of Ph+ B-ALL or ly-BC-CML patients can still experience a CNS relapse. Considering the accuracy of conventional flow cytometry (FCM) in detecting CNS disease, BCR-ABL oncoprotein determination in CSF using a cytometric immunobead assay would be particularly attractive.

For the first time, we describe the miniaturized application of the original assay to detect BCR-ABL fusion protein in CSF samples. We studied 3 patients with Ph+ B-ALL and one patient with CML in blast crisis. Although cell counts were very low (3 out of 4 investigated patients had a cell count <50 cells/μl) this approach was able to evidence the BCR-ABL oncoprotein in the 2 cases having a positive CSF result based upon conventional FCM. Our data demonstrated the FCM feasibility in detecting the BCR-ABL aberrant protein also in CSF, in spite of its very low cell numbers respect to peripheral blood (PB) and bone marrow (BM). The cytometric detection of BCR-ABL in CSF could offer a new diagnostic tool particularly in situation such as (i) Ph+ CNS isolated blast crisis in presence of a complete BM and PB cytogenetic response to imatinib, (ii) Ph-negative acute lymphoblastic leukemia occurring in imatinib treated Ph+ CML patients, and (iii) secondary Ph+ CML in patients with previous Ph-negative neoplasms.

## THE NEGATIVE SELECTION OF T LYMPHOCYTES BY IMMUNOMAGNETIC SELECTION FOR HAPLOIDENTICAL TRANSPLANTATION

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Introduction: Our study aims at demonstrating the effectiveness of CliniMACS® device (Miltenyi Biotec) for the depletion of α/β T lymphocytes from mobilized peripheral blood from healthy donors. This method retains "potential beneficial effector cells" as NK cells and γ/δ T lymphocytes. In particular, γ/δ T lymphocytes might facilitate engraftment, might have potent anti-leukemic effects and do not cause GvHD.

Methods: A total of thirteen procedures was performed. GCSF® and MOZOBIL® were used prior to leukapheresis. The immunoselection was carried out with the

automated CliniMACS® device. CD19+ cells also were depleted. Prior to and after α/β T-cells / CD19+ depletion, cells were analyzed for CD3, CD4, CD19, CD20, CD16/56, CD8, CD34 and the α/β and γ/δ T-cell receptors. Flow cytometric analysis was performed using a flow cytometer (FACSCalibur, Becton Dickinson).

Results: The mean percentage of α/β T cells prior to depletion was 28,81% (range 9,35–44,95%). After depletion, the mean percentage of α/β T cells remaining was 0.13% (range 0,01–0,60%). The mean absolute number of α/β T cells after depletion was 0,023 x 109 (range 0,004–0,098 x 109). The mean absolute number of CD19+ cells after depletion was 0,078 x 109 (range 0,004-0,233 × 109). The mean recovery of CD34+ stem cells was 90% (range 74–100%). The mean recovery of CD16+/56+ NK cells and γ/δ T cells was 77% (range 50–100%) and 83% (range 47–100%).

Conclusions: The α/β T cells depletion and the preservation of γ/δ T cells in the graft in combination with NK cells and other cell populations might offer important perspectives in a clinical transplant setting and result in significant benefits for the patient.

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## A SIMPLE AND FAST, NONDENATURING FISH LABELLING PROCEDURE ON SORTED SUPER STRETCHED CHROMOSOMES

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Fluorescence *In Situ* Hybridization (FISH) on stretched DNA of interphase nuclei, known as 'Fiber-FISH', represents an unrivaled technique for high resolution FISH, enabling detection and analysis of organization of low-copy and short DNA sequences. On the other hand, fiber-FISH does not allow localization of such sequences with respect to chromosomal landmarks, such as centromeres or telomeres, nor their association to specific chromosomes. Another, high-resolution method of analysis of mitotic chromosomes is super-stretching, through which, although a lower degree of DNA molecule extension can be reached, limitations inherent to fiber-FISH are minimized or eliminated. In fact, the integrity of chromosome structure is preserved, and after a soft treatment with proteinase K on sorted chromosomes collected onto a microscope slide, the stretching is obtained by application of Carnoy solution (ethanol:acetic acid 3:1) on the slide surface.

Here we present a new, simple and fast non-denaturing FISH method to detect DNA sequences on flow-sorted, super-stretched mitotic chromosomes of wheat. Using appropriate probes, this method allows a rapid and high-resolution analysis of sequence organization on chromosomes, as well as additional fine chromosome investigations, including structural modifications. The results will be discussed in terms of new perspectives in plant and animal chromosome analyses.

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## A NOVEL FLOW CYTOMETRIC APPROACH TO DISTINGUISH CIRCULATING ENDOTHELIAL CELLS FROM ENDOTHELIAL MICROPARTICLES

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Circulating endothelial cells (CEC) and endothelial micorparticles (EMP) are emerging as markers of endothelial repair and activation/apoptosis. Although significant changes in the number of CEC and EMP in pathological conditions have been reported, their reliable identification and quantification still remains a technical challenge. Here, we present new methodology for the identification and quantisation of CEC and EMP based on multicolour flow cytometry. Using a lyse/no wash protocol, we observed that in 50 μl of peripheral blood, the large majority of events expressing an endothelial phenotype (CD45−/CD146+/CD34+) is due to non-nucleated particles carrying mitochondrial activity and, therefore, classified as EMP. We enumerated circulating EMP by single platform absolute count in a lyse/no wash four-color flow-cytometric procedure, which allowed the distinction, within the whole endothelial compartment, of EMP derived from endothelial progenitors (CD45−/CD146+/CD34+/CD117+) and from mature endothelial cells (CD45−/CD146+/CD34+/CD117−). A significant increase in both subsets was observed in patients with diabetes mellitus. Thus, this simple and highly reproducible method maybe useful formonitoring endothelial dysfunction in clinical settings.

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## TO UNDERSTAND MSC IMMUNOMODULATION BY STUDYING THE RESPECTIVE ROLE OF MSC LICENSING AND NK ACTIVATION

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Mesenchymal stem cells (MSC) have been shown to inhibit proliferation of in vitro-stimulated lymphocytes. MSC are well tolerated when infused in vivo in allogeneic settings and tend to migrate in areas of damage or inflammation. Based on these properties, MSC have been proposed for the treatment of severe immune activation and are currently used in steroid-refractory GVHD. Although the immunosuppressive effect of MSC has been described for more than a decade, many issues remain to be solved as concern to the efficacy of these procedures. Indeed, clinical trials suggested that these treatments can have variable efficacy in different patients.

NK lysis of MSC can be one of the mechanisms involved in this variability, as it has been demonstrated that IL-2 activated NK cells are able to kill MSC. On the other hand, MSC that have been previously exposed to IFN-gamma are resistant to NK-mediated lysis, probably because of an upregulation of HLA class I on their surface. Herein decided to test if BM-MSC, untreated or pretreated with IFN-gamma, are killed by preactivated PBMC, which can best mimicry the condition of immune activation in vivo in conditions such as GVHD. MSC number and vitality will be studied by measure of impedance in the plate system xCelligence (Roche). CD107a expression on NK will be studied by flow cytometry as a correlate of NK degranulation in response to MSC. Our results should contribute to improve the knowledge of interactions between MSCs and NK cells and to improve the MSC-based therapies for severe autoimmune disorders and GVHD.

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IN VITRO ENHANCEMENT OF SAOS2 CELL CALCIFIED MATRIX DEPOSITION ONTO R.F. MAGNETRON SPUTTERED BIOGLASS-COATED TITANIUM SCAFFOLDS

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Scaffolds produced with titanium alloys (Ti-6Al-4V) are widely used as implants. To improve and accelerate the osteoin-tegration, bioactive coatings (biocoatings) of the metallic surfaces have been developed. The most studied biocoatings are based on the apatites and hydroxyapatite (HA)-based materials, because of their chemical similarity with the natural components of bone tissue. Many physical deposition methods have been tested. Among them, r.f. magnetron sputtering technique is of particular interest for the relatively low cost and the capability to deposit films of controlled stoichiometry with good homogeneity. In this study, we developed a method for the deposition of bioactive glass onto a 3D Ti6Al4V scaffold and we investigated the improved properties of the bioactive glass coated scaffolds in terms of osteoblasts morphological analysis, proliferation, and deposition of a mineralized extracellular matrix (ECM). SEM images showed that cells more homogeneously covered the surface and spanned to the neighboring fibers on the bioglass-coated scaffold than they did on the uncoated scaffold. The qualitative evaluation of the calcium deposition by calcein detection confirmed that the deposited ECM was considerably greater on the bioglass-coated 3D Ti scaffolds than on the uncoated scaffolds. The immunolocalization of bone proteins showed a more intense green fluorescence on the bioglass-coated cultured scaffolds, revealing the stimulatory effects of the bio-coating in terms of higher cell proliferation and more intense fluorescent staining of ECM. In conclusion, this study supports the hypothesis that the deposition with a simple technique of a controlled and homogeneous film of bioglass coating onto a 3D Ti scaffold enhances osteointegration.

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ELECTROSPUN FIBER MORPHOLOGY EFFECT ON IMMUNE RESPONSE

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Medical devices and tissue engineered constructs may induce an inflammatory reaction—termed foreign body reaction (FBR)—after their *in vivo* implantation. Despite recent advances in material science and tissue engineering, current knowledge of the inflammatory mechanisms associated with FBR remains scanty. Macrophage activation can be modulated by biomaterial topography according to the biological scale (micrometric and nanometric range). In this study we investigated the effect of fiber diameter (microfiber and nanofibers) and fiber alignment (aligned and random fibers) of electrospun poly(L-lactic) (PLLA) scaffolds on macrophage RAW 264.7 activation and secretion of proinflammatory cytokines and chemokines at 24 h and 7 days. SEM observation of the interaction between macrophages and different types of PLLA scaffold, revealed a greater number of normal than activated macrophages at 24 h. Elongated cells with numerous cellular projections were observed after 7 days on the micro-and nanofibrous scaffolds. On PLLA film, macrophages appeared activated both after 24 h and 7 days. To correlate the morphology of RAW 264.7 cells with viability, we investigated the percent of adherent macrophages cultured on each type of PLLA scaffold after 7 days. After 7 days, a highly significant percentage of adherent macrophage cells was observed on aligned microfibers and a slightly less elevated percentage on aligned nanofibers compared with PLLA film ($p<0.05$). In summary, our results indicate that the diameter of electrospun PLLA fibers, rather than fiber alignment, plays a relevant role in influencing *in vitro* macrophage activation and secretion of proinflammatory molecules.

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MICROCOUNT®: A NEW SIMPLE IMMUNOMAGNETIC ENRICHMENT SYSTEM FOR DIRECT COUNTING AND SEPARATION OF CIRCULATING TUMOUR CELLS

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Circulating tumour cells (CTCs) may provide new insights in clinical oncology aiming toward individualized therapy. The biological characterization of these cells will furnish additional important information. In this field, technical-methodological approaches are crucial and there is a great need for development of sensitive technologies able to avoid stress on the cells during manipulation, to preserve cell viability and proliferation capability. We describe An innovative system developed in our laboratory and recently patented (MICRO-COUNT®), which exploits the immunolabelling capture with the peculiar advantages of conventional microscopy in samples containing extremely rare cells. We focused on EpCAM +ve,cytokeratins +ve and CD45 −ve cells. The second labelling step is performed using Dynal magnetic beeds directly visible by light microscopy. MICRO-COUNTTM exploits the validated technology of magnetic bead labelling with the peculiar advantages of conventional microscopy in managing and counting a very limited number of cells. A few decades of cells recovered on a glass slide can in fact be analized and counted in a variety of a microscopical modes. The system is composed of a plate housing different types of highly efficient neodymium-magnets located in the bottom of a six well standard flask used for enrichment/separation. A square 20mm glass coverslip (located at the well's bottom) may be used to recover CTCs at the end of the procedure. This coverslip can be easily handled for further cytologi-cal characterization. Peculiar advantages of the system are: a) there is no need to stress the sample inside the standard magnetic column where the "positive selected" cells must be released by mechanical shock b) sample storage after counting for permanent case record in which further analyses (i.e. re-staining by any conventional or immuno-procedures) is allowed.

Our system allows a rapid and accurate identification/collection of viable CTCs, making possible further biological/functional characterization of the separated cells.

GLUTAMATE NEUROTRANSMISSION IS MODIFIED IN THE SOD1/G93A MOUSE MODEL OF ALS

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Glutamate(Glu)-mediated excitotoxicity plays a major role in the degeneration of motor neurons in ALS. Reduced astrocytary Glu transport was suggested as a cause. Here we report our studies on the exocytotic release of Glu as a possible source of excessive transmission. We studied the release of [3H]D-aspartate and of endogenous Glu induced by depolarizing and non depolarizing stimuli, in mice expressing human SOD1 with the G93A substitution -a transgenic model of ALS -respect to mice expressing the human SOD1 and to non transgenic littermates. Both the spontaneous and the exocytotic release of Glu induced by KCl or ionomycin was dramatically increased in symptomatic SOD1/G93A(+) mice than in controls. The higher Glu release in mutants was already present in early-symptomatic 70–90 and pre-symptomatic 30–40 day-old mice. As to the molecular determinants of this Glu exocytosis, increased $Ca^{2+}$ levels were detected in SOD1/G93A(+) mouse spinal cord nerve terminals, with an increased activation of CaM kinase II and phosphorylation of synapsin I. Moreover, release experiments suggested that the increased Glu release in SOD1/G93A(+) mice involves the readily releasable pool of vesicles and a greater capability of these vesicles to fusion upon stimulation. The reported different modulation of Glu, GABA and glycine release could induce an unbalance between spinal inhibitory and excitatory transmission in ALS.

## ONCOLOGY

TEMOZOLOMIDE INDUCED C-MYC-MEDIATED APOPTOSIS VIA AKT SIGNALING IN MGMT EXPRESSING GLIOBLASTOMA CELLS

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Purpose: We investigated the molecular mechanisms underlining the cytotoxic effect of Temozolomide (TMZ) in human O6-Methylguanine-DNA Methyl transferase (MGMT) depleted or not glioblastoma cell lines. Since TMZ is used in clinics in combination with Radiotherapy, we also studied the effects of TMZ in combination with Irradiation (IR).

Methods: Cell colony-forming ability was performed by using clonogenic assay. Cell cycle analysis and apoptosis were evaluated by Flow Cytometry (FCM). Cell cycle molecules were detected by Western blot and co-immunoprecipitation assay.

Results: Our data showed that TMZ, independent of MGMT expression, inhibited glioblastoma cell growth via an irreversible G2 block in MGMT depleted or the induction of apoptosis in MGMT expressing cells. When TMZ was administered in combination with IR, the apoptosis was greater than the one observed with the two agents used separately. This TMZ-induced apoptosis in the MGMT expressing cells occurred through Akt/Glycogen-Synthase-Kinase-3ß (GSK3ß) signaling and was mediated by Myelocytomatosis (c-Myc) oncoprotein. Indeed, TMZ phosphorylated/activated Akt led to phosphorylation/inactivation of GSK3ß which resulted in the stabilization of c-Myc protein and subsequent modulation

of the c-Myc target genes involved in the apoptotic processes. Conclusion: c-Myc expression could be considered a good indicator of TMZ effectiveness.

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IDENTIFICATION AND CHARACTERIZATION OF CANDIDATE L-ICs IN MLL-AF4+ INFANT ALL

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Recent findings have challenged the cancer stem cell hypothesis, and in particular, for ALL, the existence and the identity of a candidate leukemia-initiating cell (L-IC) is still controversial. The aim of this study was to set up an *in vivo* mouse model to identify and characterize the L-ICs in MLL-AF4+ infant ALL, the most frequent and aggressive form of leukemia occurring in patients within 1 year of life. We have assessed the leukemogenic potential of different BM subsets purified by FACS from 7 diagnostic samples and subsequently transplanted into irradiated NOD/SCID mice. The phenotype of the initial populations transplanted and the resulting leukemia in xenografts, as well as the presence of the MLL gene rearrangement was assessed by combined immunophenotype-FISH analysis. We observed that the expression of CD19 antigen is crucial to identify the L-ICs, as CD19-cells do not have the ability to initiate leukemia in mice, or give rise to normal reconstitution *in vivo*; thus suggesting that MLL-AF4+ infant ALL is more likely to originate from B-cell committed precursors. However, among the CD19+ fraction, multiple phenotypically different subsets (regardless the expression of CD34 and NG2) can serve as L-ICs in mice. Although in primary recipients the CD34+CD19+ cells appear to be the most potent and primitive L-ICs, which self-renew and give rise to the more mature CD34-CD19+ fraction, the existence of a cell hierarchy is still unclear. Our data suggest that the MLL-AF4+ infant ALL L-ICs are not confinable to a rare and distinct cell subset phenotypically defined, but rather diffuse and more heterogeneous than what initially thought. These findings have fundamental implications in therapy, and might help to unravel the dismal prognosis in this high risk leukemia.

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COMBINED TREATMENT MITOTANE/IONIZING RADIATIONS INHIBITS THE TUMOUR GROWTH IN A XENOGRAFT MODEL OF ADRENOCORTICAL CARCINOMA

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A previous study has shown that the combination of ionizing radiation (IR) and mitotane induces a growth arrest in H295R and SW13 adrenocortical cancer cells, characterized by an irreversible accumulation in the G2 phase of cell cycle.

The aim of this research is to test the effect of the mitotane and ionizing radiations singularly and in combination with them in a xenograft model of adrenocortical carcinoma (ACC). Nude mice were infected with H295R cells. Neoplastic growth was monitored by the measurement of two diameters twice a week. The development of treated and untreated tumours was monitored by MRI analyses. At the 3rd 7th, 14th day, mice were sacrificed and the tumours, were removed. In order to understand the molecular mechanisms underlying mitotane and IR effects, we analyzed cell cycle phases and DNA synthesis by FACScan cytofluorimeter. Molecules involved in the cell cycle were investigated by Western blot analysis. Finally, we assessed the cortisol level in mice.

Our data demonstrated that the rate of tumor growth inhibition in the combined treatment group was significantly greater than that in other groups. This inhibitory effect was evident by MRI, in which an intratumoral necrosis was recognizable in T2-sequences. FACScan analysis showed a G2 phase block in the early days and later a necrotic cell death in the ACC treated. Cyclin B1 and PARP expression levels, investigated by Western blotting, were deregulated after the combined treatment. Besides, the cortisol level was significantly reduced in the samples treated with IR/mitotane.

These data, *in vivo*, emphasize the inhibitory effects observed *in vitro*. Our findings support the role of combined treatment IR/mitotane in controlling ACC proliferation.

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EXPRESSION OF CD133, CANCER STEM CELL MARKER, IN A LOW GRADE TUBULOBULAR BREAST CARCINOMA: A COMPARATIVE STUDY AMONG QUANTITATIVE mRNA EXPRESSION, FLOW CYTOMETRY AND IMMUNOHISTOCHEMISTRY

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Background: CD133 marker is the elective molecule for isolation of cancer stem cells (CSCs). Recently, it has been reported that the CD133 is expressed in invasive ductal breast carcinomas. Here, we analyze CD133 expression in breast cancer by cytometric analysis, immunohistochemistry and Quantitative Real Time PCR and we evaluated if three methodologies are efficacy in detecting of CD133 marker.

Methods: Twelve patients were enrolled in this study at the National Cancer Institute of Naples. For each patient,

immunohistochemical staining for CD133, ER, PgR, c-erb B2, Ki67, E-cad, CD44 markers on embedded-paraffin tissues was performed. Real Time-PCR for CD133 on frozen biopsies was also performed. Moreover, the tissue obtained from surgery was tested by flow cytometry for CD44 FITC, CD326 PE, CD45APC and CD133PE.

Results: On 12 patients, 6 showed high expression (>66%), 3 low (<66%) and 3 no expression of estrogen receptor. The expression of progestin receptors was high (>66%) in 5 cases, low (<66%) in 2 cases, not expressed in 5 cases. The expression of Ki67 was high (>20%) in 7 cases, and low (<20%) in 5 cases. The E-cad was expressed in 11 cases. For CD133 and CD44 on tissue samples, the percentage of CD133+ cells was very heterogeneous. In 5 samples, the expression was very low or absent, in 5 it was moderate (5-15%), in one case it was >20% and only in Tubulobular variant breast cancer, it was about 35%. CD44 expression was also extremely variable. By flow cytometry, the mean percentage of CD133 positive cells was 13,75% and the mean percentage of CD44 positive cells was 17,90%. All cells analyzed were positive for CD326 (EpCAM). In Tubulobular breast variant, the percentage of CD133 was about 70%. Both immunohistochemistry and cytometric data for CD133 were confirmed by Real Time-PCR.

Conclusion: We have evaluated the rise of CD133 antigen as a suitable marker for CSCs detection in breast carcinomas. The analysis was carried out by different methods, flow cytometry, immunohistochemistry and Real Time PCR quantification. Our data were confirmed by all three methods, and the results are overlapping.

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ELECTROPORATION INCREASES ANTITUMORAL EFFICACY OF THE BCI-2 ANTISENSE G3139 AND CHEMOTHERAPY IN A HUMAN MELANOMA XENOGRAFT

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Background and aim. Nucleic acids designed to modulate the expression of target proteins remain a promising therapeutic strategy in several diseases, including cancer, but clinical success is limited by the lack of efficient intracellular delivery. Here, we evaluated whether electroporation (EP) could increase the delivery of antisense oligodeoxynucleotides against bcl-2 (G3139) as well as the efficacy of chemotherapy in human melanoma xenografts.

Methods: Melanoma-bearing mice were treated with G3139 and/or cisplatin (DDP) followed by the application of EP to tumors. Protein and mRNA expression were analyzed by Western blot, immunohistochemistry and realtime PCR. Tumor apoptosis and proliferation index were analyzed by TUNEL and Ki67, while G3139 tumor accumulation was measured by Flow cytometric analysis and confocal microscopy. Antitumor efficacy was evaluated in terms of tumor weight inhibition (TWI) and tumor regrowth delay (TRD).

Results: The G3139/EP combined therapy produced a significant TWI (more than 50%) and a marked TRD (about 20 days). The efficacy of this treatment was due to the higher intratumoral concentration of G3139 than treatment with oligos alone, as showed by FACS analysis, which led to a marked down-regulation of bcl-2 protein expression. Moreover, the G3139/EP treatment resulted in an enhanced apoptotic index and a decreased proliferation rate of tumors. Finally, an increased tumor response was observed after treatment with the triple combination G3139/DDP/EP, showing a TWI of about 75% and TRD of 30 days.

Conclusions: These results demonstrate that EP is an effective strategy to improve the delivery of antisense oligodeoxynucleotides within tumor cells *in vivo* and it may be instrumental in optimizing the response of melanoma to chemotherapy.

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PRELIMINARY STUDY IN EARLY LUNG CANCER DETECTION USING SPUTUM SAMPLE

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Lung cancer is the leading cause of death worldwide. There is a lot of evidence that evaluates the relationship between chronic bronchitis and lung cancer. Chronic obstructive pulmonary disease (COPD) refers to chronic bronchitis and emphysema, a pair of two commonly coexisting diseases of the lungs in which the airways become narrowed. The leading cause of both lung cancer and COPD is well recognized in tobacco use. The focal point of our study is to assess the capability of Image Cytometry to identify preneoplastic lesions that occur in smokers using 5cER as diagnostic parameter that could help clinicians in early identification of lung cancer. In total, the sputum of 80 smokers, aged 50–80 years, was collected. Out of the total, 79% had a diagnosis with typical diagnostics procedure (both COPD of various grade than lung cancer), 21% had no definitive diagnosis. In the first case, 5cER value confirmed both cancer and no-cancer diagnosis with sensivity and specificity of 60% and 80%. In the second case, identification of lung cancer or no lung cancer patients had a sensitivity and specificity of 67%. Moreover, our aim is to identify predictive markers and to understand if they have a

correlation with ploidy status. Preliminary data show that same genes have positive correlation ($r > 0,5$) and same negative correlation ($r < -0,5$). The goal of our study is to increase the number of samples to improve our preliminary statistics.

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## IMMUNOPHENOTYPIC, IMMUNOCYTOCHEMICAL, CYTOGENETIC AND MOLECULAR ANALYSES OF A RARE TYPE OF SARCOMATOID PULMONARY BLASTOMA

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We analised a rare primary malignancy of the lung, classified as Biphasic Pulmonary Blastoma, characterized by the presence immature cells with epithelial and mesenchymal features.

Immunocytochemical, immunophenotypic, cytogenetic and molecular analyses were performed both on tumor tissue and on corresponding Pulmonary Blastoma derived cell line in order to characterize the possible epitelial-mesenchimal transition process in the contest of Cancer stem cell-like (CSCs-like). CSCs or initiating tumor cells are cell subpopulations with stem cells properties. Such cell populations were characterized by Flow Cytometry analysis, thus determining cell immunophenotype. Recently, research works have found some relationships between CSCs model and epithelial-mesenchymal transition in cancer thus to understand their potential role in the processes of invasion, metastasis and mobilization.

Cytological investigation performed on blastoma cell line, showed two different populations in morphology: polygonal and elongated fibroblast-like. These populations when studied by flow cytometry, confirmed the presence of both the epithelial and mesenchymal cells, therefore, showing the progressive decrease of the epithelial marker CD326 and the increase of the mesenchymal marker CD90, while culturing. Moreover, it was really interesting the increasing cell expression of CD133, a CSCs specific marker in derived cell lines monitored by FCM analyses.

Blastoma tissue sample showed two DNA Index different populations: 1.00 and 1.40; while tumor-derived cell line, was characterized by the progressive loss of diploid cell population and by the positive selection of aneuploid cells showing 1.4 DNA Index. The positive selection of aneuploid cell line was also confirmed by molecular cytogenetic investigations performed by FISH technique. FISH analysis showed a tumor cell line genome characterized by numerous structural and numerical chromosomal aberrations: tetrasomy of chromosome 8 and an 8q isochromosome; tetrasomy of chromosome 4 with Prominin1 gene (CD133); Y cromosome disomy. The instability of the tumor genome was confirmed by LOH analysis using the technique of polymorphic STR markers. QPCR with STR marker was performed on tissue sample, and on obtained cell line, in order to investigate the progressive loss of heterozigosity spanning the entire tumor genome. Fifteen STR markers were tested, and the analysis revealed the progressive LOH from the tumor sample to the cultured and selected cell line. At least a molecular analysis by direct sequencing was performed for the K-Ras and p53 genes, in order to investigate possible gene mutations. Those molecular target were chosen because they play an early (K-Ras) and a late (p53) role trough cancerogenesis process. Sequence analyses revealed both in tumor tissue and in cultured cell lines the absence of mutations thus expressing a wild type target sequence. Our results suggest a new approach to study these rare malignant tumors.

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## MicroRNA PROFILING AS PREDICTIVE FACTOR OF COMPLETE PATHOLOGICAL RESPONSE TO NEOADJUVANT CHEMO-RADIOTHERAPY IN PATIENTS WITH LOCALLY ADVANCED RECTAL CANCER

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Background: MicroRNAs (miRNAs) are small, noncoding, RNA molecules involved in regulation of several cellular mechanisms. Specific miRNA have been found abnormally down-regulated or up-regulated in colorectal cancer and associated with prognosis or response to treatments. However, no study addressed their predictive role in rectal cancer. Therefore, we used microarray technology and RT-PCR to profile miRNA expression in patients (pts) affected by locally advanced rectal cancer, with the aim to identify a specific "signature" which correlates with pathological complete response to neoadjuvant chemoradiotherapy. Methods: 38 pts with locally advanced rectal cancer (uT3-4/N+) were treated with pre-operative capecitabine+oxaliplatin and pelvic conformal radiotherapy (45 cGy) followed by surgery (after 6–8 weeks). Pathologic response was scored according to the tumor regression grade (TRG) scale. MiRNA expression profile was analysed by microarray on fresh frozen biopsies, collected before treatment start, and confirmed by RT-PCR. The correlation between miRNA expression profile and the TRG, coded as TRG1 (pathologic Complete Response-pCR) versus TRG >1 (no pCR), was assessed by statistical analysis methods specifically designed for this study. Results: 14 miRNAs were selected by arrays analysis as differentially expressed in TRG1 pts and 13 of them were confirmed by RT-PCR. In particular, 11 miRNAs (miR-1183, miR-483-5p, miR-622, miR-125a-3p, miR-1224-5p, miR-188-5p, miR-1471, miR-671-5p, miR-1909*, miR-630 and miR-765) were significantly up-regulated in TRG1 pts, while 2 miRNAs were down-expressed (miR-1274b and miR-720). miR-622 and miR-630 showed 100% sensitivity and specificity for TRG1 cases and were significantly correlated to EGFR ($\chi^2$

= 11·793; p = 0.001) and TS expression ($\chi^2$ = 10.589; p = 0.001). Conclusions: A set of 13 miRNA is significantly correlated with pathologic complete response and might be considered a specific marker of response to pre-operative chemoradiotherapy in locally advanced rectal cancer.

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ROLE OF NUCLEAR LOCALIZATION OF BCL-2 PROTEIN IN THE MODULATION OF HIF-1/VEGF AXIS IN MELANOMA CELLS UNDER HYPOXIC CONDITION

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We previously demonstrated that the antiapoptotic protein bcl-2, through its BH4 domain, promotes Hypoxia-Inducible Factor 1 (HIF-1)-mediated Vascular Endothelial Growth Factor (VEGF) expression in hypoxic cancer cells and that nucleus-localized bcl-2 overexpressed protein is able to interact with HIF-1α. Using M14 cell line and its derivative clones stably overexpressing bcl-2 deleted in its BH4 domain, we found that the lacking of BH4 domain abolishes the protein-protein interaction of bcl-2 with HIF-1α and the capability of overexpressed bcl-2 to localize in the nucleus. Moreover, by using stable clones expressing *wild* type bcl-2 or bcl-2 targeted in different cellular compartments, we found that if bcl-2 protein is specifically overexpressed in the cytoplasm, it lost the capability to synergize with hypoxia to induce HIF-1α protein and HIF-1 transcriptional activity. On the other hand, transient overexpression of molecular chaperone FKPB38 in *wild* type bcl-2 overexpressing clone determined a decrease of nuclear bcl-2 protein level and reduced hypoxia-induced HIF-1a protein expression. Finally, using stable clones derived from M14 cell line transfected with a vector expressing the Flag-tagged BH4 sequence of bcl-2, we found that this domain of bcl-2 is sufficient to enhance the expression of HIF-1α protein expression, HIF-1-dependent transcriptional activity and VEGF protein secretion under hypoxic condition.

These results demonstrated that bcl-2 nuclear localization is required for bcl-2-mediated induction of HIF-1/VEGF axis, suggesting a rationale for the design of new therapeutic approaches targeting BH4 domain and/or the nuclear localization of bcl-2 to counteract melanoma progression and bcl-2 nuclear expression as a possible marker of aggressiveness in melanoma.

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GSK2118436, EFFECTIVE BRAF INHIBITOR FOR MUTATED MELANOMA CELL LINES IN BRAF V600-E

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Background: Recent data suggest new promising strategies for the treatment of patients with advanced melanoma. The most common single activating mutation in B-RAF (V600E) is found at all stages of melanoma including 70% of patients with metastatic melanoma. The aim of this study is to determine the effect in vitro of BRAF inhibitor, GSK2118436 on human BRAF V600E-mutated and Wild Type melanoma cell lines.

Materials and Methods: To evaluate the selectivity of the drug, two melanoma cell lines were tested: mutated M14 in BRAF V600E and WT-LB. To determinate viability of the melanoma cell lines, the cells were plated 2 × 104 in 96-well plates at increasing concentrations (0.5, 1, 3, 5, 10, 30, 100 nM) of the GSK2118436 molecule for 72 hrs and tested by MTT assays. Cell lines were treated with concentrations of 3, 5, 10, 30 e 100 nM for 72 hrs, and then these assays were performed: 1) staining with propidium iodide for cell cycle analysis; 2) flow cytometry for apoptotic cell death upon double-staining with Annexin V and propidium iodide; 3) western blot to evaluate the effect of BRAF inhibitor to MAPK pathway.

Results and Discussion: For cell viability, the IC50 values using GSK2118436 were 100nM for LB and 5nM for M14. Analysis of cell cycle showed a block in the phases SG2M and a major percentage of cells positive for apoptosis, for the two lines respectively to IC50 same value. Western Blotting showed a ERK phosphorylation increasingly from 3nM to 100nM. GSK2118436 might be potent inhibitors for melanoma for the target therapy.

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IDENTIFICATION OF A NEW BREAST CANCER STEM CELL PHENOTYPE ($CD44^+/CD24^{hi}/CD338^{hi}$) IN A MODEL OF BASAL-LIKE TUMOR SUBTYPE

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Background and Aim: Breast cancer is the first human carcinoma for which a putative cancer stem cell subpopulation (BCSC) has been isolated with CD44+/CD24−/low phenotype. The aim of this study was to identify new breast cancer stem cell markers. We focused our work on basal-like subtype that seems to be derived from a developmental stage of mammary epithelial cell that is different from the primitive mammary stem cell and is sometimes referred to as the luminal progenitor.

Methods: We used polychromatic flow cytometry to characterize breast cancer cell lines resembling different tumor molecular subtypes and cell sorting to isolate the subpopulations of interest. Sorted subpopulations were further studied in order to explore their stem properties through mammosphere assay, western blot analysis and *in vivo* tumorigenicity assays.

Results and Conclusions: We identified a subpopulation of BCSCs, with the $CD44^+/CD24^{hi}/CD338^{hi}$ phenotype,

within the basal-like breast cancer continuous cell line HCC1937. These cells are capable to grow in anchorage-indipendent conditions as spherical organoids. Particularly, $CD24^{hi}$ cells are able to form mammospheres with higher efficiency than CD24-cells and, among $CD24^{hi}$ cells, CD338 overexpressing cells are able to form mammospheres with higher efficiency than CD338-cells. We also found that injection of NOD/SCID mice with HCC1937 cell line lead to the formation of a tumor that is strongly enriched in expression of CD338. These data suggest that in HCC1937 cell line there is a BCSC subpopulation overexpressing CD338 and bearing the phenothype $CD44+CD24^{hi}$, i.e. an antigenic combination different from the classical $CD44+/CD24-/^{low}$.

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## DIFFERENTIATION OF Caco-2 CELLS REQUIRES BOTH TRANSCRIPTIONAL AND POST-TRANSLATIONAL DOWN-REGULATION OF Myc

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Caco-2 cancer cell line is widely used to reproduce *in vitro* the differentiation of human intestinal epithelium. This cell line, when cultured over confluence for 21 days, spontaneously undergoes cell cycle arrest and differentiates with the formation of a polarized enterocyte-like monolayer. During this process, Myc protein is completely down-regulated, as occurs in normal enterocytes. Caco-2 cells differ from normal enterocytes for mutations of APC and β-catenin genes, factors known to be involved in the transcriptional control of MYC gene during enterocyte differentiation. In this work, we investigated how Myc regulation could be achieved during Caco-2 differentiation process, notwithstanding the APC and β-catenin mutations. We highlighted the post-translational regulation of Myc protein as one of the essential mechanisms that allows the exit from cell cycle and onset of differentiation of Caco-2 cells. Moreover, we found a strong correlation between Myc protein downregulation and the expression of the transcription factor Cdx2, suggesting the existence of a regulative link between these two proteins.

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## LAMIN A/C IS REQUIRED FOR SHSY5Y NEUROBLASTOMA CELL DIFFERENTIATION AND IS LINKED TO A LESS MALIGNANT PHENOTYPE

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Neuroblastoma is one of the most aggressive tumors in childhood. The degree of its differentiation is related to patient outcome.

A-type lamins are filament proteins forming a meshwork under the nuclear membrane and they are involved in the developmental differentiation. Their expression is reduced in different human cancers although their role in tumorigenesis is not yet clarified.

Our aim was to study the role of Lamin A/C in the differentiation and tumorigenesis of neuroblastoma.

We used the SHSY5Y neuroblastoma cell line able to differentiate *in vitro* and showing high levels of Lamin A/C. As differentiating agent we used the retinoic acid (RA). Silencing of Lamin A/C blocked the RA-induced differentiation, thus preventing neurites formation and differentiation markers expression. The genome-wide gene-expression profiling of Lamin A/C silenced (miRL) cells confirmed the down-regulation in miRL cells of those genes needed for differentiation. As well, an increase of tumor progression related genes in miRL cells is associated to augmented migration and invasion abilities and to higher drug resistance. Getting insight into the mechanism underlying the higher miRL cells aggressiveness we analyzed the functionality of the nuclear pore complex and showed altered nucleocytoplasm trafficking.

Characterization of neuroblastoma by lamin A/C expression may predict the response to therapy, allowing development of new therapeutic strategies based on the tumor profiling.

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## *TEL-AML1* DEREGULATES THE CYTOSKELETON AND BLOCKS THE CXCR4/CXCL12 AXIS IN *IN VITRO* MODELS OF PRE-LEUKEMIA

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The t(12;21) chromosome translocation is a frequent initiating event in childhood leukemia, generating a clinically silent pre-leukemic B cell progenitors (BCP). In addition to the consequent *TEL-AML1*fusion gene, further second events (occurring years later) are required for overt disease.

Aim of the study is to investigate how *TEL-AML1* expression can sustain this covert condition for many years. In particular we question if the fusion gene alters the interaction of BCP with the microenvironment, important source of survival signals.

The study is performed by using two murine models: a *TEL-AML1* inducible expression system on pro-B BaF3 cell line and preBI primary cells stably transduced with pMIGR1-*TEL-AML1*-IRES-GFP construct.

In these models the introduction of *TEL-AML1* alters the expression of genes regulating the cytoskeleton, causing

changes in cellular morphology and phenotype. In particular BaF3 cells acquire long extensions and, in both models, adhesion molecules are disregulated. Moreover, *TEL-AML1* cells show a significant defect in the chemotactic response to CXCL12, although the expression and the recycling of CXCR4 receptor are unaffected. This inability is associated with a defect in CXCR4 signaling: calcium flux and ERK phosphorylation by CXCL12 stimulation are inhibited.

We are now investigating whether these alterations can affect the interaction of *TEL-AML1* pre-leukemic BCP with the microenvironment and contribute to the pathogenesis of the disease.

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## CPTH6, A NOVEL Gcn5/pCAF HISTONE ACETYLTRANSFERASE INHIBITOR, INDUCES BOTH APOPTOSIS AND AUTOPHAGY IN HUMAN LEUKEMIA CELLS

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Recent studies strongly support the histone acetylation as a promising therapeutic target. A growing body of evidence demonstrates a direct relationship between histone acetyltransferases (HAT) activity and development or progression of cancer disease. Our study identified the thiazole derivative 3-methyl-cyclopentylidene-[4-(4-chlorophenyl)thiazol-2-yl)hydrazone (CPTH6) as a novel inhibitor of HAT enzymes Gcn5 and pCAF, while it did not affect p300 and CBP activities. CPTH6 treatment significantly decreased H3 and H4 histones and □-tubulin acetylation in U937 human acute myeloid leukemia cells. Moreover, it reduced cell proliferation and activated apoptosis. Induction of autophagy was also evidenced by the formation of acidic vesicular organelles and the accumulation of both microtubule-associated protein 1 light chain 3 (LC3)-II and p62/SQSTM1 proteins. The involvement of downstream targets of Akt/mTOR pathway was observed after CPTH6 exposure, which determined 4EBP1 protein level reduction and eIF4E protein phosphorylation. Besides, combined treatment of U937 cells with CPTH6 and the mTOR inhibitor Temsirolimus determined a higher cytotoxic effect than the exposure to the single agents. Overall, these results identify CPTH6 as a new epigenetic active compound, targeting HAT and inducing both apoptosis and autophagy in leukemia cells.

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## CENTROMERE REPOSITIONING IN CLINICAL CASES AND EVOLUTION

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The evolutionary history of chromosomes can be tracked by the comparative hybridization of appropriate BAC clones. This approach has disclosed an unprecedented phenomenon: centromere repositioning, that is the movement of the centromere along the chromosome without marker order variation. Evolutionary New Centromeres (ENC) occurrence is relatively frequent. In macaque, for instance, 9 out of 20 autosomal centromeres are evolutionarily new; in donkey at least 5 such neocentromeres originated after divergence from the zebra, in less than 1 million years. Recently, orangutan chromosome 9, considered to be heterozygous for a complex rearrangement, was discovered to be an ENC. Human clinical Neocentromeres (HN) are ectopic, analphoid centromeres seeded in chromosomal acentric fragments allowing for their mitotic survival as supernumerary chromosomes which result in abnormal phenotypes. They originate as opportunistic events, secondary to a chromosomal rearrangement. The latter circumstance has been regarded as strong evidence of their epigenetic nature. In humans, in addition to this type of neocentromeres, 8 centromere repositioning events have been reported. These real time seeding events provide clues to ENC birth and progression. In addition, they suggest that ENC and human clinical neocentromeres are two faces of the same coin.

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## CD133 AND CD44 CELL SURFACE MARKERS DO NOT IDENTIFY CANCER STEM CELLS IN PRIMARY HUMAN GASTRIC TUMOURS

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Emerging evidence suggests that tumours contain and are driven by a cellular component that displays stem cell properties, the so-called cancer stem cells (CSCs). CSCs have been identified in several solid human cancers, however, there are no data about CSCs in primary human gastric cancer. By using CD133 and CD44 cell surface markers we investigated whether primary human gastric cancers contain a cell subset expressing stem-like properties and whether this subpopulation has tumour-initiating properties in xenograft transplantation experiments.

We examined tissues from 44 patients who underwent gastrectomy for primary gastric cancer. The tumourigenicity of the cells separated by flow cytometry using CD133 and CD44 surface markers was tested by subcutaneous or intraperitoneum injection in NOD/SCID and nude mice.

Gastric cancers included in the study were intestinal in 34 cases and diffuse in 10 cases. All samples contained surface marker-positive cells: CD133+ mean percentage 10.6% and CD133+/CD44+ mean percent-age 27.7%, irrespective

of cancer phenotype or grade of differentiation. Purified CD133+ and CD133+/CD44+ cells, obtained in sufficient number only in 12 cases, all intestinal type GC, failed to reproduce cancer in two mice models. However, the unseparated cells produced glan-dular like structures in 70% of the mice inoculated.

In conclusion, although CD133+ and CD133+/CD44+ were detectable in human primary gastric cancers, they neither expressed stem-like properties nor exhibited tumour-initiating properties in xenograft transplantation experiments.

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COMPARISON OF THE MODE OF ACTION AND ANTITUMOR ACTIVITY OF TRABECTEDIN AND ITS TWO DERIVATES, ZALYPSIS® AND PM01183

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Zalypsis® and PM01183 are two trabectedin (T) analogous that bind the minor groove of DNA. The aim of this study was to compare the mode of action and the *in vitro* and *in vivo* activity of T, Zalypsis® and PM01183. Like T, PM01183 showed a decreased cytotoxicity of approximately 4−5 times in NER deficient (−) cell lines compared with NER proficient (+) ones, whereas for Zalypsis® was of 1.5−2 times. The cellular pharmacokinetic of these compounds was different. At the end of 1 hour exposure with IC50, the intracellular concentrations were 0.28 pmol/106cells, for T, 1.8 pmol/106cells for Zalypsis® and 0.13 pmol/106cells for PM01183. At 2 hours after drugwashout the intracellular concentrations were 0.28 pmol/106cells for T, 0.83 pmol/106cells for Zalypsis® and undetectable for PM01183. In $NER^+$ all the three drugs induced a progressive accumulation of cells in G2M phase of the cell cycle, while in $NER^-$cellular systems did not. Small quantitative differences of antitumor activity of T and PM01183 was found in the different *in vivo* preclinical tumor models, whereas the spectrum of activity of Zalypsis® was different. These data indicate that there are important differences in the mode of action, pharmacokinetic and antitumor activity of Zalypsis® and PM01183 compared to T. These differences justify the clinical development of all three compounds that might have a different pattern of antitumor activity and toxicity.

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IDENTIFICATION OF A HUMAN NEUROBLASTOMA CELL LINE-DERIVED SUB-POPULATION WITH COLONY FORMING POTENTIAL AND SELF-RENEWAL PROPERTIES

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Neuroblastoma is an extracranial tumor that develops in children and is often a fatal disease with a survival rate of 40%. The recurrence of these tumors after chemotherapy has been partly explained by the presence of a subpopulation of tumour initiating cells (TICs). These cells share phenotypic and functional characteristics with normal stem cells, most notably their ability to self-renew and to differentiate. Using the SK-N-BE(2) cell line as a cellular model of neuroblastoma, we tested their capacity to survive in suspension using a neurosphere selective medium. We found, by both immunocytochemistry and flow cytometry, that neurosphere subpopulations derived from SK-N-BE(2) cell line specifically express nestin, SSEA-1, and a significant positivity to Aldefluor®, whereas the bulk population does not. In another experiment we identified a subpopulation of Hoechst 33342 negative cells that were sensitive to verapamil: this population counted around 0,5% as compared to all the analyzed events, whereas in neurospheres it was around 3%. Using SSEA-1 (an extracellular antigen) and the Aldefluor® reagent as positive selective markers, we sorted $SSEA\text{-}1^+/ALDH^+$, $SSEA\text{-}1^+/ALDH^-$, and $SSEA\text{-}1^-/ALDH^+$ cells by fluorescence activated cell sorting and cultivated them in neurosphere selective medium. We noticed that only the $ALDH^+$ fraction was able to reform neurospheres after two days in culture, while $ALDH^-$ cells were not, demonstrating a differential ability for self-renewal for this specific neurosphere-derived subpopulation. Moreover, neurospheres demonstrated a significantly higher capacity to form colonies as compared to the bulk population, confirming the presence of cells with an increased potential to grow without adhesion survival signals. In conclusion, we characterized a population derived from SK-N-BE(2) human neuroblastoma cell line with stem-like properties that has the ability to self-renew and to form colonies to a greater extent than the integral population.

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EPITHELIAL TO MESENCHYMAL TRANSITION BY TGF-1 INDUCTION INCREASES STEMNESS CHARACTERISTICS IN PRIMARY NON SMALL CELL LUNG CANCER CELL LINE

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Background: Cancer Stem Cells (CSCs) hypothesis asserts that only a small subset of cells within a tumour is able of both tumour initiation and sustainment. The Epithelial-Mesenchymal Transition (EMT) is an embryonic developmental program that is often activated during cancer invasion and metastasis. The aim of this study is to shed light on the relationship between EMT and CSCs by using LC31 lung cancer primary cell line.

Materials and Methods: A549 and LC31 cell lines were treated with 2ng/ml TGFβ-1 for 30 days, and 80 days, respectively. In order to evaluate EMT, morphological changes were assessed by light microscopy, immunofluorescence and cytometry for following markers: cytokeratins, e-cadherin, CD326 (epithelial markers) and CD90, and vimen-

tin (mesenchymal markers). In addition, RT-PCR for Slug, Twist and β-catenin genes were performed. On TGFβ-1 treated and untreated LC31 cell lines, we performed stemness tests such as pneumospheres growth and stem markers expression such as Oct4, Nanog, Sox2, c-kit and CD133. Western Blot for CD133 and tumorigenicity assays using NOD/SCID mice were performed.

Results: TGFβ-1 treated LC31 cell line lost its epithelial morphology assuming a fibroblast-like appearance. The same results were obtained for the A549 cell line (control cell line). Immunofluorescence and cytometry showed up-regulation of vimentin and CD90 and down-regulation of cytocheratin, e-cadherin and CD326 in TGFβ-1 treated LC31 and A549 cell lines. Slug, Twist and β-catenin mRNA transcripts were up-regulated in TGFβ-1 treated LC31 cell line confirming EMT. This cell line showed also over-expression of Oct4, Nanog, Sox2 and CD133, all genes of stemness. In addition, an increased pneumosphere-forming capacity and tumours-forming ability in NOD/SCID mice were detected in TGFβ-1 treated LC31 cell line respect to cell line untreated.

Conclusions: The induction of EMT by TGFβ-1 exposure, in primary lung cancer cell line results in the acquisition of mesenchymal profile and in the expression of stem cell markers.

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GROWTHINHIBITORY EFFECTS OF SCD40L ON COLORECTAL CANCER CELLS

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The cell surface costimulatory molecule CD40 has been indicated as prognostic/predictive biomarker and as a potential target for therapy in human tumours. The CD40 pathway regulates humoral and cellular immunity, exherts direct antiproliferative effects on selected tumours and plays a role in angiogenesis. This multifunction nature of the CD40 signalling depends on target cells, on microenvironment and on different mechanisms of stimulation (ligation or crosslinking). Discordant results have been reported about the growth-inhibitory effects and the potential for inducing apoptosis of the two forms of the CD40 ligand: soluble (sCD40L) and membrane bound. We studied three previously established colon cancer cell lines, well-characterized for CD40 expression: Colo320 (moderate expression), HCT116 and SW48 (highly positive). To investigate the growth inhibitory mechanism of the sCD40L we evaluated its effect on cell cycle phase distribution and apoptosis induction. Cell cycle analysis was performed on Propidium Iodide (PI)-stained cells and apoptosis was assessed by Annexin V-FITC/ Propidium Iodide assay, by flow cytometry (FCM). CD40 antigen was expressed on 8% of Colo320 cells and 32% and 53% of SW48 and HCT116 cells, respectively. 48 hrs after incubation with sCD40L the studied cell lines were markedly ($p < .005$) accumulated in G0/G1 phase with a significant ($p < .05$) decrease of cells in S-phase, compared to untreated cells. At the same time point after treatment no significant apoptosis was observed in all the studied cell lines. In our study the inhibition of colorectal cancer cell proliferation by sCD40L was mainly due to a slowing down of the cell cycle progression while apoptosis was not involved in the growth inhibition. This finding is in contrast with recent reports on CD40 +ve colorectal cancer cells. These data should be taken into account when the CD40 pathway is utilized as a therapeutic target, in view of a possible combination of standard chemotherapy and/or antiangiogenetic therapy with antitumour immunotherapy in advanced colorectal cancer.

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ALDEHYDE DEHYDROGENASE EXPRESSION AND EPITHELIAL STEM CELL PHENOTYPE IDENTIFY CLONOGENIC TUMOUR CELLS IN HEPATOCELLULAR CARCINOMA

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In vitro colonyforming capability is one of the principal characteristics of Cancer Stem Cells (CSCs), which are now considered to have a leading role in the onset and the development of tumours. The phenotype of hepatocellular carcinoma (HCC) CSC still remains elusive. The aim of this study was to characterize CSCs in 3 different cell lines (hcc-1, -2, -3), generated in our laboratory from XXIX Conferenza Nazionale di Citometria Centro Congressi Grand Hotel Salerno 58 ottobre 2011 Salerno a single human HCC specimen, by means of single immunophenotyping, clonogenicity and aldehyde dehydrogenase (ALDH) activity. Clonal efficiency and ALDH expression were different in the three cell lines, and were reciprocally related (i.e. 36% and 90% in hcc-3; 17% and 50% in hcc-1; and 11% and 5% in hcc-2). Cells isolated by fluorescence activated single cell sorting for the expression of the principal stem cell markers (i.e. two epithelial stem cell markers: EpCAM for all the three cell lines and CD56 for hcc-3; two typical mesenchymal markers: CD44 for hcc-1 and Thy-1 for hcc-2) were evaluated for colony formation. We found that hcc-1 CD44 negative, hcc-2 Thy-1 negative and hcc-3 CD56 positive cells were more clonogenic than their counterparts ($p = 0.0006$, $p < 0.0001$ and $p = 0.0001$, respectively) and with a high ALDH activity. Regarding EpCAM expression, positive cells were slightly more clonogenic than the negative ones only in hcc-1 (56.5% vs. 47.4%, $p = 0.03$) but they showed variable expression of ALDH.

Our data suggest that ALDH expression associated with epithelial phenotype and no markers of epithelial to mesenchymal transition is a better marker than EpCAM alone for the identification of a more clonogenic cell population. Phenotypically different clonogenic tumour cells can be found in the same human HCC.