# XXV CONFERENZA NAZIONALE DI CITOMETRIA

## *La Citometria nell'era delle Biotecnologie*

**Pontificia Università Lateranense, Roma-Città del Vaticano**
**3-6 ottobre 2007**

# PROCEEDINGS OF THE XXV NATIONAL MEETING

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

## October 3 - 6, 2007
## Rome-Vatican City

Following the first experience in 2005, also this year an issue of Cytometry is partly dedicated to the programme and abstracts of the National Conference of the Italian Society of Cytometry, GIC.

The XXV edition of the Conference has been organized in October 2007 in Rome-Vatican City.

Abstracts selected by the Scientific program Committee are published here in full and categorized by scientific track.

The 2007 Conference, with the title "Flow Cytometry in the Biotechnology Era", was the main event celebrating the 25 years since the foundation of the Society.

Following a continuous growth in these years, to date there are over 845 members actively involved in educational programs, promotion of quality controls programs and drafting/validation of guidelines, providing information for people involved that actively work in the field of basic and applied cytometry.

This year 152 contributions have been selected by the Scientific Committee among those submitted by basic and clinical researchers operating in the various Italian Institutions.

The Conference and its scientific production provided a comprehensive idea as regard the status of translational and clinical research in the various aspects of cytometry in Italy, paying particular attention to the young researchers and their efforts in this specific field.

Many investigators have submitted high quality data that were organized (as 57 oral and 95 poster presentation) inside specific sessions. Oral presentation included pre-Congress seminars designed by the Scientific program Committee to be of the highest scientific merit. Experts in the field served as Chairmen to place the findings into perspectives. The Conference had been opened by a talk dealing with the story of Cytometry in Italy including the founding of the GIC Society. The ISAC President Paul Robinson made an overwiev of the analytical capability of flow Cytometry in the "cytomics generation".

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

In addition, different topics of general interest in biological and medical sciences, new data on the study of Cell Cycle and Apoptosis by flow cytometry and on the Methodological and Technological advances were reviewed by experts from Italy and, for the specific field of the Cell Therapy, from USA.

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

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

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

Guest Editors:
R. De Vita - G. Mazzini

Marco Danova
GIC President

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## INVITED SPEAKERS

### TWENTY-FIVE YEARS OF CYTOMETRY IN ITALY: OUR CONTRIBUTION TO ITS DEVELOPMENT

**G. Mazzini**
*IGM-CNR, Pavia Italy*

The GIC Meeting in Rome 2007 has a particular meaning since it signs the twenty-five years after the initial activity of the "founder group" of the Society. Many research groups in Italy were already engaged in programmes dealing with cell analyses as well as in quantitative microscopy and some of us have been impressed (attending international Meetings) by the "hot technology" of the moment. Among others, the automated cell analyses have been the topic able to fuse together experiences coming from different scientific area such as medical, biological, physical, engineering .... all dreaming on the possibility to improve the "healthcare" with instruments capable to made automated diagnoses especially for cancer pathology. The dreams still remain largely unsolved, but a great improvement of the knowledge in these field has been acquired. Scientists were split in two groups, one believing on the cell analyzed on smears by means of "image analysis" and the other one dealing with cells forced to flow in a capillary.

In Italy since the nineteen eighties a group of young researchers starting to be involved in both the "streams" (the large part interested in "flow") decided to give a "structure" to an informal community of people already active in the field. Few years later, thanks to the enthusiastic action of Francesco Mauro, the "Italian Group of Cytometry GIC" was found in Rome the 29 of September 1986. The Society headquarter was established inside the Biomedical Dept. of ENEA (Rome) still housing the Secretary and "the life" of the Society. The beginning of the "GIC story" was characterized by a very active and enthusiastic participation of a large number of young members involved, together with the founders, in the organization of the initial events. We all were positively impressed by some international cytometry Meetings (the Elmau series, as well as the "historical" - not only for science - but also for the unusual cold-storm-weather with snow in Bracciano) unforgettable milestones in the story of cytometry worldwide. The DNA quantitation and relative biomedical involvements dealing with the crucial establishment of "normality" and "abnormality" (detection of "cytometric aneuploidy") drive the initial GIC activity. Other than "seminars" and practical Courses a series of "National trials" on this theme had been organized with a great respond of participants all around italy. The scientific value of the work done had been reported in a paper published in Cytometry (14, 1993 and 30, 1997b). The GIC Council also promote another national trial in the area of immunopheno-typing again with a very large success in terms of partecipants involved. The results of the hard work have been also published in Cytometry (14, 1993). The Council of the Society, already composed by members from different scientific area, decided to constitute some sub-committees as "Working Groups" dealing with the different application areas of cytometry. The GIC activity started to play an important role in teaching and up-grading the young applicants and students thus organizing (alternatively to the annual Meeting) the Annual Courses covering both the basic as well as the advanced needs of the people involved in cytometry. The continuous growth of the Society (now based on more than 300 regular members) suggested the establishment of a permanent teaching structure we called "National School of Cytometry" largely supported, at the beginning, by the facility of the Urbino University and now very well spread all around Italy from Milan to Palermo. The future of the Society is looking in the contact and cooperation with parental societies at any national and international level: many years ago we pioneered a joint intersociety Meeting with the French Society (Lerici 1988) and now the GIC President welcomes in Rome other colleagues from few important national societies all interested in established or in the new applications of cytometry.

### CYTOMETRY AND THE DAWN OF THE CYTOMICS GENERATION

**J. Paul Robinson**
*SVM Professor of Cytomics, Purdue University President, International Society for Analytical Cytology*

Cytometry has for many years focused on traditional techniques that are well understood and provide a quality of information on single cells, not possible with other technologies. However, as all technologies change, so to is cytometry. There is a gently but strong move toward tools that are driven by the demands of more complex systems biology approach. The result is a more comprehensive field of cytomics broadly defined as the systematic study of biological organization and behavior at the cellular level has begun to mature and establish itself as an integral component in cell biology. The necessary tools for integration of cytomics into the fundamental nature of cell systems analysis are maturing but new tools are demanded to achieve our goals. While there is a long way to go before we have tools that can perform true cytomics analysis, cytometry is a subset of tools that is tremendously powerful and from which we can extract a significant subset of information about many biological systems.

It is important therefore to realize that new technologies must be developed for cytomics to become a reality.

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For example there will be a need for essential development of new sensor technologies that provide both sensitivity and selection in the visible and near IR spectrum. Secondly, a better integration between different measurement and detection tools will be needed. We simply cannot make independent measurements and hope to integrate these tools easily. Thirdly, in order to analyze the complex data sets resulting from new technology integration a major advance is needed to accommodate analysis of these data sets. Fourthly, chemistries must advance to permit greater selectivity of tracking tools. These will most likely expand beyond fluorescence to accommodate enhanced scatter analysis as well as chemical composition.

Together, these advances place the Cytomic opportunity into a new dimension for understanding metabolic responses in single cells and ultimately defining new functional populations of cells. The result will be new research tools as well as a toolset for clinical and diagnostic utility.

## MICROARRAY-CGH AND GENOME PLASTICITY

**Mariano Rocchi**

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The human genome has been fully sequenced since 2001. In the meanwhile other entire genomes have been sequenced, and their comparison started to delineate our recent evolution. Few data, however, were available on genome variability among humans. The HapMap project has partially filled the gap. The microarray technique, initially developed for gene expression purposes, turned out to be very efficient also in determining DNA gain and losses in tumors (array-CGH, Comparative Genome Hybridization). During 2005, the microarray-CGH was utilized, for the first time, to investigate differences among individuals. Other studies followed. The surprising results revealed an unprecedented level of copy number variation among humans and started to identify genes showing copy number polymorphisms and that were involved in susceptibility to diseases.

## OPTICAL FLUORESCENCE NANOSCOPY

**Alberto Diaspro**

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Optical microscopy plays a key role in bioimaging as it allows exploring nature in a multidimensional way within the continuing development of new approaches that are in tune with the 1959 Feynman's sentence: "there is a plenty of room at the bottom". Optical microscopy is rapidly moving to nanoscopy exploiting the running multiphoton revolution that brought a dramatic and wide-reaching change in bioimaging. Considering only a part of the field of application, say cell imaging, one has to consider that the size of biological molecules operating and interacting in cellular systems varies dramatically, from small fatty acids and sugars ($\approx$ 1 nm = 10–9 m), to proteins (5–10 nm), starches (>1000 nm), and the enormously elongated DNA molecules. Within this framework, optical microscopy is still unique in allowing to explore the 3D space occupied by biological systems - from macromolecules to cells, from tissues to organs - while temporal changes occur within a temporal scale from microseconds to several hours and days. It's worth outlining currently developing approaches down to the nanoscale within a 7D observation window: from 3D to time until spectral information (5D) plus lifetime (6D) and high-order harmonics (7D). 3D is the starting point: from computational issues (from image deconvolution to fuzzy approaches) to architectural solutions (from fast confocal to multiphoton high resolution interactions). FRAP and FRET methods are part of it. In particular, the advent of two-photon excitation (2PE) of fluorescence has led to terrific advances. Optical 2PE approaches from microscopic level to single molecule imaging can be tailored for specific experimental needs. Moreover, the coupling with new fluorescent molecules, including photoactivatable and photoswitchable ones, makes the "microscopical machine" an enormously powerful tool in bioimaging.

## PLASMACYTOID DENDRITIC CELL LYMPHOMA

**Luigi Del Vecchio**

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During the last decade an unusual form of lymphoma whose cells expressed HLA-DR, CD4, CD56 and IL3R was described. Functional and phenotypic features of these cells were similar to the lymphoplasmacytoid subset of normal dendritic cells. The clinical behavior of this disease generally implies skin involvement with an aggressive outcome and possible picture of acute leukemia. Two main types of patients have been described: (i) cases with exclusive bone marrow involvement and (ii) cases with initial skin involvement and subsequent leukemic dissemination. In order to ameliorate our approach to this disease, it is necessary (i) to define the exact immunophenotype of this rare neoplasia, (ii) to assess the real incidence of these cases, (iii) to attempt an immunophenotypic subclassification. In our experience, flow cytometry characterization has consistently evidenced the phenotype HLA-DR+, CD4+, CD56+, CD36+, CD103+. Generally, cases with bone marrow involvement are initially referred to our institution as acute myeloblastic leukemia (AML). In patients with cutis involvement, immunohistochemistry (IH), performed on cutaneous biopsies, consistently evidenced expression of CD4, CD56, CD123 and TCL1, along with variable expression of CD68 and CD43. TdT was variable by both flow cytometry and IH. CXCR4 was observed by IH in all cases while convincing expression of CD26 was difficult to find.

Immunophenotype of this rare disease seems to be stable as regards HLA-DR, CD4, CD56, CD36 and CD103 display. CXCR4 and CD26 appear to be associated with tissue distribution of neoplastic cells, with CXCR4+CD26- pattern corresponding to cases characterized by initial cutaneous

involvement and metastatic potential, CD26 bright expression being associated to bone marrow disease.

## IMMUNOPHENOTYPIC ANALYSIS OF MYELODYSPLASTIC SYNDROMES

**Matteo G. Della Porta**

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The pathologic hallmark of myelodysplastic syndromes (MDS) is marrow dysplasia, which represents the basis of the WHO classification of these disorders. The combination of overt marrow dysplasia and clonal cytogenetic abnormality allows a conclusive diagnosis of MDS, but this is found in only a portion of patients. In many instances, the diagnosis is exclusively based on morphological criteria.

Flow-cytometry immunophenotyping is a reliable method for quantitative and qualitative evaluation of hematopoietic cells. Its application the setting of MDS initially showed abnormal expression of various surface antigens. Several studies have then evaluated the clinical utility of this approach, suggesting that flow cytometry might provide useful information in management of MDS patients.[1–4]

Abnormal maturational pattern in the granulocytic series was frequently reported in MDS,[1] together with irregular expression of CD10, CD33, CD56 and CD64 on neutrophils and monocytic cells[1,3] and with asynchronous/aberrant expression of myeloid and lymphoid-associated antigens on CD34+ progenitor cells.[5] Assessment of erythroid dysplasia represents a challenge in the immunophenotypic analysis because of the limited availability of specific antibodies. Cytometric analysis of iron metabolism proteins was demonstrated to expand the potential of immunophenotyping in this setting. In addition, evaluation of mitochondrial ferritin might become the standard approach to diagnosis of sideroblastic anemia.[4]

Although no single immunophenotypic marker proved able to discriminate accurately between MDS and other pathological conditions, multiparametric approaches allowed to correctly classify a high proportion of MDS.[1,3,4] Moreover, immunophenotyping was demonstrated to be informative in a significant percentage of cases with a non-conclusive morphological analysis.[1,3,4] Among MDS patients classified according to WHO criteria, an isolated involvement of the erythroid lineage is associated with a better prognosis than that of multilineage dysplasia.[6] Thus, the possible recognition of dysplasia by immunophenotyping with higher sensitivity and specificity may have important prognostic significance.

A critical point of the morphological evaluation of marrow dysplasia is that inter-observer reproducibility is often inadequate, particularly in low-grade MDS. Flow cytometry can provide better reproducibility than morphology[1,3] Mean fluorescence intensity analysis appears to be of particular interest and was successfully tested in a multicentric laboratory setting.[2,4]

In conclusion, immunophenotyping may provide an accurate evaluation of marrow myeloid and erythroid dysplasia and might be considered for the implementation of the WHO diagnostic criteria for MDS. Multicentric prospective studies are warranted to standardize cytometric analysis and to validate immunophenotyping in the diagnostic work-up of MDS patients.

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## PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) IN THE ECULIZUMAB ERA: THE BEDSIDE AND BEYOND

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PNH is a hematological disorder characterized by clonal expansion of hematopoietic stem cell(s) lacking all surface GPI-linked proteins due to *PIG-A gene* mutation; as a consequence, progeny RBCs lacking the complement regulators CD55 and CD59 undergo complement-mediated chronic intravascular hemolysis. Eculizumab (Soliris®, EC) is a humanized mAb against C5, which inhibits the terminal MAC formation. We have collected clinical and experimental data from 21 Italian transfusion-dependent PNH patients enrolled within the EC-based international trials TRIUMPH, SHEPHERD and Extension. All patients showed blockade of intravascular hemolysis, pointed out by a striking LDH level reduction, which resulted in beneficial clinical responses (cessation or reduction in transfusion requirement in 76% and 19%, respectively); these findings were associated with an expected increase in circulating PNH RBCs, as demonstrated by serial flow cytometry analysis. C3 coating on RBCs, WBCs and Plts was investigated by flow cytometry, using anti-C3 polyclonal Abs in combi-

nation with anti-CD59 or against lineage specific GPI-linked proteins. RBCs from healthy controls were always CD59+/C3− RBC from patients with cold agglutinin disease were mostly CD59+/C3+ (positive control). Untreated PNH patients had both CD59+/C3− and CD59−/C3− and never C3+ RBCs. During EC treatment all 21 patients showed a substantial proportion of CD59−/C3+ RBCs and never CD59+/C3+ RBCs. C3 was also found on neutrophils in most patients, and on Plts in some. We hypothesize that EC inhibits intravascular hemolysis, but does not oppose C3 accumulation on RBC surface as a consequence of CD55 absence, possibly leading to extravascular hemolysis through the reticulo-endothelial system via complement receptors; this may explain the suboptimal clinical response in some patients.

## BONE MORPHOGENETIC PROTEINS INHIBIT THE CANCER-INITIATING ABILITY OF HUMAN GLIOBLASTOMA STEM-LIKE CELLS

**Angelo Vescovi**

*DIBIT-Fondazione San Raffaele Milano*

Transformed, stem-like precursors, which establish tumors in vivo, have been identified in human brain cancers. We report that bone morphogenetic proteins (BMPs), amongst which BMP4 elicits the stronger effect, activate their cognate receptors (BMPRs) and trigger the Smad signalling cascade in cells isolated from human glioblastomas (GBMs) and enriched for tumor stem cells. This is followed by a reduction in proliferation and increased expression of differentiated neural markers, without affecting cell viability. A reduction in the clonogenic ability, in the size of the CD133+ population and in the growth kinetics of GBM cells indicates a BMP4-mediated reduction in the stem cell population. Notably, the action of BMP4 greatly reduces the capacity of GBM cells to establish GBMs upon intracerebral transplantation. Most important, in vivo delivery of BMP4 effectively blocks the tumor growth and associated mortality, that occurs in 100% of the control mice in less than twelve weeks, following intracerebral grafting of human GBM cells. Thus the BMP-BMPR signalling system, which regulates the activity of normal brain stem cells, is also a key regulatory component of GBM tumor stem cells and represents a candidate target for the treatment of incurable astrocytomas.

## ISOLATION AND CHARACTERIZATION OF CANCER STEM CELLS FROM SOLID TUMORS

**Ruggero De Maria**

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Cancer stem cells are the rare population of undifferentiated tumorigenic cells responsible for tumor initiation, maintenance and spreading. Such population should represent the preferential target of effective therapies aimed at eradicating the tumor. The development of technologies that allow the unlimited in vitro expansion of cancer stem cells could be a powerful tool for basic and translational researchers aimed at studying the pathogenic events that drive cancer initiation and progression, while providing crucial information for the development of new therapeutic compounds targeting specific survival pathways of the tumorigenic population.

We isolated and expanded tumorigenic cancer stem cells from a variety of solid tumors, including thyroid, brain, lung, colon and breast cancer. Upon injection in immunocompromized mice, these cells are able to reproduce the original tumor, as assessed by both morphological and molecular analysis. We observed that the analysis of basic biological parameters concerning cancer stem cells may provide a powerful tool to determine the prognostic value in the clinical setting. The evaluation of glioma stem and progenitor cell frequency into the tumor mass, together with their chemotherapy resistance and enhanced growth, are able to clearly identify the patients at higher risk of disease progression and death. Likewise, the study of cancer stem cells from other tumors allowed us to successfully test preclinical models of cancer therapy. Thus, although the identification of cancer stem cells from solid tumors is very recent, this research area appears extremely promising and able to foster novel diagnostic and therapeutic applications in experimental and clinical oncology.

## ''IT'S A SMALL WORLD'' CYTOMETRIC DETECTION AND CHARACTERISATION OF BACTERIA

**Gerhard Nebe-von-Caron**

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Image cytometry has been with us for more than 300 years starting with the detection of single bacteria. In 1883 the first flow cytometer based on light scatter measurement was used to prove that deterioration (putrefaction) of nutrient solutions did not start spontaneously but required particles. Microbiology was then the first science based on single cell analysis using the colonies growing on solid nutrient support as developed by Koch.

Modern day cytometry is focussed on direct single cell measurements allowing the analysis of cells that can not be cultured either because they are injured or their culture requirements are too difficult. Because they can measure several parameters at once they reveal heterogeneity not only in complex mixtures but even in apparently homogeneous cultures.

As it is critical to ensure that the measured events are bacteria, problems with signal intensity and signal discrimination will be discussed as well as the problems of interpreting light scatter data.

The labels already included in some of the marine samples give a message about function and allow some classification in combination with light scatter. Ribosomal probes, lectin stains and immuno-fluorescence are the classical way of differentiation on the single cell level examples of the latter two in combination with functional measurements will be given

Fluorescent protein expression as markers for cellular processes has become a recent target for cytometric investigations. Observing gene expression at the single cell level demonstrates the shortfall of the bulk measurement approach as will be shown in examples of salmonella and for the germination of a bacillus.

Cell function is one of the key measurement criteria. Interpretation of the fluorescent stains is difficult as probes do not necessarily behave as published. A "reasonable" approach has to be taken to explain the staining patterns obtained. Measurements can be taken to differentiate viability into reproductive growth, metabolic activity and cell integrity. Problems of differential staining and functional assessment will be discussed with examples of injured aggregates and the detection of the living dead.

## FLOW CYTOMETRY AND MARINE MONITORING FOR THE EARLY WARNING OF BIOLOGICAL RISKS

**Raffaella Casotti**

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Outbreaks of marine toxic and/or harmful organisms are increasing in time and intensity as a result of ecosystem modifications due to climate changes. These represent a dangerous risk for the human health and economics, mainly in coastal regions where marine products are used for recreational or consumption purposes. Invasive species from ballast waters or from human transportation may also represent a biological hazard often only detected after damage is made. Flow cytometry occupies a first row in the monitoring of the marine environment to detect changes related to climatic variability and for the early alerts of toxic blooms and associated biological risks. New developments in flow cytometry for environmental studies aim at miniaturization and automation as well as at realtime transfer of data. For these, submersible instruments are developed and deployed in key areas during critical seasons. A review of the present instruments commercially available and of the prototypes developed for research reveals a wealth of diversity in type of cells to be analyzed, and also in applications. One main field is the monitoring of coastal areas for the early detection of toxic plankton organisms. Apart from typical measurements of scatter and fluorescence, innovative sensors allow the detection and characterization of the pulse shape generated by chains or colonies and other optical properties that can help identification. New hybrid instruments have coupled either molecular biological techniques, such as DNA microarray, or microscopy to implement the capacity of flow cytometers to analyze seawater samples and are currently employed to detect specific organisms. Real-time warning relies on transmission of data through satellite or radio-communication. Data can be transmitted remotely and used to alert the population or to stop exploitation, e.g. of shellfish at specific locations. In summary, flow cytometry offers unique possibilities in the monitoring of potentially hazardous biological phenomena and is becoming a very valuable tool in the aquatic field.

## RE-THINKING CELLULAR PHARMACODYNAMICS

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Despite the impressive advance in molecular biology, the methods to assess anti-proliferative drug effects in vitro are still those of twenty years ago, scoring the macroscopic variations on measurable quantities as growth inhibition or flow cytometric percentages of cells in $G_1$, S and $G_2M$. As the end-result is the superimposition of the effects of cell cycle block and cell loss, their time-course before the measure, and the proliferation of surviving cells, each measure conveys only a piece of information, that may be misinterpreted if taken individually.

To decode this complex system we set up an approach based on in silico simulation of cell cycle progression as a function of the $G_1$, S, and $G_2M$ durations and of their perturbations, described by parameters representing $G_1$, S and $G_2M$ checkpoint activities, and enabling to fit together all the data, obtained with different techniques, like static or flow cytometry or antiproliferative tests. By the comparative analysis of several anticancer drugs we suggest that the overall response of all drugs is the result of the combination of few types of checkpoint response, which operate with different strengths, and with specific drug concentration thresholds.

Our approach suggests a way to go beyond pharmacodynamics models based on proportionality between instantaneous concentration and effect, towards a re-foundation of cellular pharmacodynamics accounting for the complexity of the underlying cell cycle interactions.

## THE CONTRIBUTION OF BIOTECHNOLOGY TO THE DEVELOPMENT OF NEW DRUGS

**Marco Renoldi**

*a.d. Amgen SpA, Vice-Presidente Assobiotec*

An effective pharmacological medication is currently available for no more than 500 diseases, while known diseases are more than 10,000. Biotechnology plays a larger and larger role in driving medical research towards meeting unmet medical needs: biological drugs include already some of the most innovative therapies against grievous diseases such as cancer, autoimmune diseases, CNS disorders, diabetes and chronic kidney disease. 50% of all drugs currently in development are biotech in nature.

Recombinant DNA technology was the foundation in the development of the first biological drugs, mostly hormones and cytochines. Newer technologies are currently being probed to provide more precise, hence effective and safe medications. Today's key challenge is identifying therapies that specifically target the individual genetic code.

The new frontier of biotechnology research and development is systemic biology, i.e., the science which integrates genetic, genomic, biochemical, cellular, physiological and clinical data to establish a database network, which in its turn can be leveraged to model predictive biological events and therefore yield new discoveries and potentially revolutionary medications.

Biotechnology-driven innovation can successfully fulfil a series of unmet medical needs. However, innovation comes with a price. Growing healthcare costs, also influ-

enced by demographic trends and the introduction of innovative medications, require payers to set stringent access rules for innovative biologicals. In the attempt to demonstrate not only the safety and efficacy but also the value of new drugs, the next challenge is the identification of predictive efficacy markers for new biologicals, so that proposed use is more precise and social cost more acceptable.

## AIDS 2007. WHAT IS NEW

**Roberto Cauda**

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During the past year, several important randomized clinical trials of antiretroviral therapy have been published or reported at the relevant international meetings in the field of AIDS. These have expanded the available options of protease inhibitors as first line recommended treatments from the initial lopinavir/r to fosamprenavir/r and atazanavir/r, while for saquinavir/r ongoing clinical studies still need longer term confirmatory results. Moreover, new active drug options for multi-drug experienced patients carrying resistant virus have become available, including protease inhibitors with higher genetic barrier, such as tipranavir and darunavir. Agents from other classes, such as promising second generation NNRTI-inhibitor etravirine and drugs from 2 new classes, the integrase inhibitor raltegravir and the CCR5 coreceptor antagonist maraviroc have shown very convincing results and are now available through large phase IIIb studies. Other promising agents from these and other classes are at earlier developmental stages. The long term safety and efficacy data of these agents will help to define their optimal combination and sequential use, in order to fine-tune and also individualize safe and effective life-long treatments.

The field of opportunistic infections and neoplasms registers on the one hand a continuous reduction, but also still worrying high prevalences because of AIDS-presenting patients, which still account for substantial AIDS-related morbidity and mortality throughout European countries. Often a shift towards tuberculosis as the most prevalent opportunistic infection is observed, which underscores the need for better surveillance and prevention strategies. New data also highlight the increasing prevalence of non-HIV related morbidity and mortality among HIV-infected patients. Given the ageing of the HIV-infected population, the longer survival and the presence of several co-factors, liver disease, mostly due to HCV, and non-HIV related neoplasms, in particular lung and anal cancer, are increasing their relative incidence. Since the incidence of these disorders are related to the immunodeficiency levels, they may, in the near future, drive decisions towards an earlier initiation of anti-HIV therapy, also in the light of the decreasing toxicity of newer antiretroviral agents.

## BASE EXCISION REPAIR IS DOWNREGULATED IN TERMINALLY DIFFERENTIATED MUSCLE CELLS: A CLUE FOR HYPERSENSITIVITY OF MYOTUBES TO OXYGEN INJURY

**Laura Narciso,[1] Paola Fortini,[1] Deborah Pajalunga,[1] Anna Paola Franchitto,[1] Pingfang Liu,[2] Paolo Degan,[3] Mathilde Frechet,[4] Bruce Demple,[2] Marco Crescenzi,[1] and Eugenia Dogliotti[1]**

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The differentiation of skeletal myoblasts is characterized by permanent withdrawal from the cell cycle and fusion into multinucleated myotubes. The muscle cell survival is critically dependent on the ability of cells to respond to oxidative stress. Base Excision Repair (BER) is the main repair mechanism of oxidative DNA damage. In this study we compared the levels of endogenous oxidative DNA damage and BER capacity of mouse proliferating myoblasts and their differentiated counterpart, the myotubes. A change in the regulation of key genes of the antioxidant response was observed during differentiation with an increase in the intracellular free oxygen radical concentration and 8-hydroxyguanine DNA levels in myotubes as compared with myoblasts. The repair of 2-deoxyribonolactone that is exclusively processed by long patch BER was impaired in cell extracts from terminally differentiated muscle cells. The repair of a natural abasic site that is a preferential substrate for short patch BER was also delayed in myotubes. The defect in BER of terminally differentiated muscle cells was ascribed to the nearly complete lack of DNA ligase I and to the strong downregulation of XRCC1 with subsequent destabilization of DNA ligase IIIalfa. The attenuation of BER efficiency in myotubes was associated with a significant accumulation of DNA damage as detected by nuclear foci containing phosphorylated histone H2AX upon exposure to hydrogen peroxide. We propose that in skeletal muscle, under exacerbation of free radical injury, the accumulation of unrepaired repair intermediates, due to attenuated BER, might contribute to myofiber degeneration as seen in sarcopenia and in many muscle disorders.

## T REGULATORY CELLS BETWEEN AUTOIMMUNITY AND TUMOUR IMMUNITY

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This presentation discusses briefly the viewpoint that autoimmunity and tumour immunity are two sides of the same coin and that a subtle balance exists between pathogenic and beneficial immunity. In the context of autoimmunity, it is likely that following external assault, (parts of)

damaged cells are taken to regional lymph nodes by professional tissue antigen presenting cells (APC). In the lymph nodes, both CD4+ and CD8+ damaged cell-specific, tissue-homing, resting cytotoxic T lymphocytes (CTL) as well as regulatory T cells (including natural $CD4^{+}CD25^{high}$Foxp3+ T cells and inducible IL-10 and TGFβ producing Tr1 cells) will be activated through crosstalk between different subsets of APC and T cells, with the help of appropriate costimulatory signals. In this way, the normal situation of self tolerance to autologous cells is over-ridden. As a result, both humoral and cellular immunity develop. Moreover, in the effector phase, dendritic cells can phagocytose the dying cells and present cell-derived antigens to T cells that are recruited in response to chemokines produced by tissue due to stress (incidentally, this is the typical situation occurring after cytoreductive chemo- and radiotherapy). Thus, dendritic cells are the sentinels that decide whether tolerance or self-destruction must occur. Dendritic cells, continuously interact with naturally occurring CD4+ $CD25^{high}$Foxp3+ T cells (thereafter referred to as Tregs), engaged in the maintenance of immunological self-tolerance and suppressive control of aberrant or excessive immune responses to foreign antigens. This interaction may, on the other hand, impedes immune surveillance against cancer and hampers the development of effective immunity to autologous tumor cells. Indeed, Tregs have been observed to predominantly infiltrate tumor masses especially in the early phase of tumor progression. Several animal data indicate that depletion of Tregs by removing CD25 expressing T cells prior to tumor challenge is able to provoke effective tumor immunity. Furthermore, elimination of Tregs or attenuation of Treg-mediated suppression in ongoing anti-tumor immune responses, for example by altering signaling through some surface molecule involved in suppression (namely, CTLA-4 or GITR) can enhance the anti-tumor response and contribute to eradicate advanced cancers. Thus a combination of depletion or attenuation of Tregs and concomitant stimulation of effector T cells, systemically or locally in tumors, can be regarded as a feasible immunotherapy for cancer.

## CELL CYCLE AND APOPTOSIS

### CHARACTERIZATION OF LAK CELL MEDIATED CYTOTOXIC MECHANISMS VERSUS TUMORAL TARGETS

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NK and T cells become lymphokine-activated killer cells (LAK) after appropriate cytokine stimulation. LAK cell activity against tumor cells can occur via a $Ca^{++}$-dependent perforin-mediated necrosis or perforin/granzyme-mediated apoptosis, alternatively $Ca^{++}$-independent apoptosis can be induced via TNF ligand members. A traditional cytometric assay for cell-mediated cytotoxicity, requires the uptake of propidium iodide (PI) by target cells, however it cannot discriminate between apoptosis and necrosis (1). To this regard, we have developed an assay that utilizes the green fluorescent dye, DIOC18, to label tumor targets and the supravital PI (50 μg/ml) staining to evaluate target cell lysis and apoptosis (2). Leukemic Jurkat cells were co-incubated with LAK cells at different target:effector (T:E) ratios. In some experiments, EGTA, a calcium chelating agent, or AAD and VAD inhibitors were also used to selectively block the $Ca^{++}$-dependent mechanism, or granzyme-B and caspase pro-apoptotic activities, respectively. Our results show that the assay allows to evaluate both Jurkat cell necrosis and apoptosis. As expected, EGTA blocked Jurkat lysis, while caspase or granzyme-B inhibitors significantly reduced apoptotic cell death. Interestingly, apoptosis was prevalent at low T:E ratio. Since in vivo the T:E ratios are likely low, we suggest that apoptotic cell death rather than inflammatory necrosis, should preferentially occur during in vivo cell mediated cytotoxicity.

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### IN VITRO STUDY ON THE MECHANISM OF ACTION OF LAURYL GALLATE IN HUMAN CULTURED CANCER CELLS

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Lauryl gallate is an antioxidant food additive showing low toxicity to normal cells and good specificity to tumor cells. The effect of lauryl gallate was firstly evaluated on three human breast cancer cell lines: MCF7, expressing wt p53; multidrug-resistant MCF7 ADR and MDA-MB-231 cell lines which have mutant, not functional, p53. Cell viability assay demonstrated that lauryl gallate induced a dose- and time-dependent decrease of viable cell number. Cell cycle alterations (stable arrest in $G_1$ phase in MCF7 and a delay in cell cycle progression in the other two cell lines) were revealed by flow cytometry. Lauryl gallate caused an upregulation of p21 in the three breast cancer cell lines and an increased expression of p53 only in MCF7, as shown by western blotting. Induction of apoptotic program was evaluated by annexin V-FITC, Hoechst labeling, analysis of PARP cleavage and ultrastructural alterations. Lauryl gallate treatment induced

activation of Erk1/2 MAPK, which in turn was responsible for upregulation of p21, cell cycle alteration and apoptosis. The inhibition of Erk1/2 activation by PD98059 (inhibitor of the upstream kinase Mek1/2 activity) attenuated lauryl gallate-induced cytotoxicity. These findings strongly suggest that Erk1/2 activation mediated growth inhibition and apoptosis induced by lauryl gallate, through a p53-independent mechanism. Additional experiments showed that this compound also inhibited cell proliferation of other human tumor cell lines, which carry not functional wt p53 (M14, melanoma) or mutant p53 (PC3, prostate carcinoma; LN229, glioblastoma). Growth inhibition assay and annexin V-FITC labeling showed that PC3 and M14 cell lines were more sensitive to the treatment than glioblastoma cells. This antioxidant compound might be a good candidate for innovative therapeutic strategies against tumors with p53 mutations and resistant to conventional chemotherapeutic agents.

## DISSIPATION OF MITOCHONDRIA ΔΨm: A FLOW CYTOMETRIC DETECTION BY DIFFERENT DYES

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Mitochondrial permeability transition, the collapse of electrochemical gradient across the mitochondrial membrane (ΔΨm), is a hallmark of cell death. The measurement of mitochondrial membrane potential is a valuable method to study signaling mechanisms involved in the initiation of the apoptotic cascade. In the present communication we compare different dyes to study this process by cytofluorimetric methods and assess the mitochondrial membrane function and structure. In the first step of our study, we performed analyses on fresh and aged peripheral blood cells (PBCs). PBCs were labelled by means of JC1, TMRE, MitoTracker Green (MTG) and the new redoxsensor probe, CC1. The fluorescence localization of the latter probe appears to be based on a cell's cytosolic redox potential and accumulates both in mitochondria and lysosomes. TMRE and CC1 flow cytometric staining was performed by optimising protocols used in fluorescence or confocal microscopy. Flow cytometry was performed in single and dual parameter manner. We found that double staining with TMRE/MTG was particularly valuable as a tool to investigate mitochondrial status. Furthermore, in this double staining the distribution of events on cytometric dot plots was interpretable in a simple manner and showed both similarities and differences with the green/red staining of the popular JC1 probe.

## A NOVEL METHOD BASED ON CLICK CHEMISTRY COUPLING, WHICH OVERCOMES LIMITATIONS OF CELL CYCLE ANALYSIS BY CLASSICAL DETERMINATION OF BrdU INCORPORATION, ALLOWING MULTIPLEX ANTIBODY STAINING

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Determination of BrdU incorporation into DNA is a widely used method to assess the cell cycle status of cells. DNA denaturation is required for BrdU detection with the drawback that most epitopes of proteins are destroyed and classical antibody staining methods for multiplex analysis are not possible. To address this issue we setup a novel method for measuring cells in active S-phase that overcomes the DNA denaturation step but still detects BrdU. For this purpose cells were pulsed for a short time by an alkynyl deoxyuridine, which is incorporated into DNA. The nucleotide exposed alkyne group from DNA was then derivatized in physiologic conditions by Azide-Alkyne Huisgen 1,3-dipolar-copper (I)-catalyzed cycloaddiction reaction ("Click Chemistry" coupling) using a BrdU azide derivative (5-Bromo-5′-azido-2′,5′-dideoxyuridine). The resulting DNA-bound bromouracil moiety was then detected by commercial Anti-BrdU monoclonal antibodies without the need for a denaturation step. This method has been tested on several cell lines and is superior compared to traditional BrdU detection because it allows multicolor and multiplex analysis in flow and image cytometry, as exemplified. A comparison between direct coupling by fluorochrome (Alexa$^{TM}$ 488) azide and our indirect BrdU Azide-based method has been performed. This method will open new opportunities to investigate simultaneously changes in protein epitopes or expression during the cell cycle.

## SPATIAL DISTRIBUTION AND CO-LOCALIZATION OF p21$^{CDKN1A}$ AND PARP-1 IN BASE EXCISION REPAIR

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The cell cycle inhibitor p21$^{CDKN1A}$ plays a fundamental role in several aspects concerning the DNA damage response, from cell cycle arrest, regulation of transcription, apoptosis, and possibly DNA repair. The interaction of p21 with poly(ADP)ribose polymerase-1 (PARP-1) and PCNA suggests an involvement in base excision repair (BER). Here we have investigated whether p21 is relevant to the BER process by analysing the cellular effects of the alkylating drug, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), in p21$^{-/-}$ human fibroblasts. We have found an increased sensitivity to MNNG-induced DNA damage, and a delayed kinetics of recruitment of PCNA and PARP-1 to DNA damage sites, in p21$^{-/-}$ as compared with p21$^{+/+}$ fibroblasts. Interestingly, p21 co-localised with PARP-1 at the nucleolar level, suggesting a specific interaction at this site. Analysis of the PARP-1 region involved in the binding to p21 was performed in HeLa cells by co-expressing PARP-1 full

length, or N- and C-terminal fragments fused to GST, together with p21-GFP. Confocal microscopy analysis and affinity pull-down experiments showed that the C-terminal region of PARP-1 was associated to p21-GFP, thus indicating this domain responsible for the interaction.

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MECHANISMS OF ACTION OF *MALALEUCA ALTERNIFOLIA* (TEA TREE) OIL ON *CANDIDA ALBICANS*

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The essential oil of *Malaleuca alternifolia* (TTO) exhibits broad-sprectum antimicrobial activity. Here, we report the effects of TTO treatment against the yeast *Candida albicans.* Proliferation study showed that TTO was effective both on fluconazole-susceptible (3153) and on -resistant isolate (AIDS68) strains of *C. albicans.* The cells of the two strains were grown in the presence of TTO at the concentration ranging from 0.25% to 1% for 2, 10, 30 and 60 min. The analysis by fluorescence microscopy after Hoechst staining revealed an evident chromatin condensation in 3153 cells at 2 min, while in AIDS 68 cells it appeared at 30 and 60 min. Other morphological alterations typical of apoptosis, such as nuclear fragmentation, was never observed at any time of treatment. In order to clarify the mechanisms of action of TTO, we analyzed the cell cycle of the same samples by flow cytometry (FASC) after propidium iodide staining. DNA histogram analysis revealed that TTO treatment induced an accumulation in $G_1$ phase. Moreover, antifungal effect was detected by FACS analysis of PI-treated cells. TTO did not increase the rate of PI-positive yeast cells. These preliminary findings seem to indicate this natural product as a new antimycotic agent with a special mechanism of action.

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OXIDATIVE STRESS IN *CANDIDA ALBICANS*

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Cells respond to oxidative stress by inducing the expression of protective proteins, by repairing stress-related damage or by inactivating reactive oxygen species (ROS). In this study we have analyzed the ROS production in strains of *Candida albicans,* as related to the susceptibility of fungal cells to antifungal agents. Thus, the fluconazole (FLU)- and micafungin (FK)-susceptible strain (CO23s) was cultured in increasing concentrations of each drug to generate fluconazole-resistant $CO23_{RFLU}$ and micafungin-resistant $CO23_{RFK}$ strains, then the cells were treated with $H_2O_2$ (50mM) and analyzed by rhodamine 123 (Rh123) accumulation. This analysis revealed a notable increase of ROS levels in oxidant-treated CO23s and $CO23_{RFK}$ cells while no alteration of ROS levels was found $CO23_{RFLU}$ treated cells. The same results were also obtained after treatment of the three strains of *C. albicans* with the antifungal drugs fluconazole and micafungin. Moreover, biochemical analysis of glutathione (GSH) levels revealed a higher GSH content in $CO23_{RFLU}$ than in CO23s and $CO23_{RFK}$ strains.

These preliminary results suggest that the low sensitivity of $CO23_{RFLU}$ yeast cells to antifungal drugs might involve resistance mechanisms to oxidative stress, probably mediated by the high GSH content.

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$O_{2/3}$ EXPOSURE INHIBITS CELL PROGRESSION AFFECTING CYCLIN B1/CDK1 ACTIVITY IN SK-N-SH WHILE INDUCES APOPTOSIS IN SK-N-DZ NEUROBLASTOMA CELLS

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In search for innovative therapeutic agents for children neuroblastoma, the oxygen therapy could be considered an alternative antitumoral treatment. Given the physio-chemical properties of $O_{2/3}$ gas mixture including fairly low aqueous solubility and spreading, and the interesting perspective of hyperoxia, we analyzed the inhibitory effect of $O_{2/3}$ treatment on two human neuroblastoma cell lines (SK-N-SH and SK-N-DZ). In this study we demonstrated that $O_{2/3}$ treatment was able to induce cell growth inhibition and cell cycle perturbation in both cell lines. We observed an arrest at $G_2$ phase, accompanied by an alteration in the expression and localization of cyclin B1/cdk1 complex and a reduction in its activity in SK-N-SH cells. This reduction was consistent with the increase in both Wee1 and chk1 protein levels. On the contrary, $O_{2/3}$ induced apoptosis in SK-N-DZ cells *via* caspase 3 activation and Poly ADP-ribose polymerase-1 (PARP) cleavage, associated with an increase in the pro-apoptotic Bax protein. Consequently, we considered the possibility of improving the responsiveness to chemotherapeutic agents such as Cisplatin, Etoposide and Gemcitabine in combination with $O_{2/3}$ treatment. The combined treatments produced a stronger cell inhibitory effect than Cisplatin and Etoposide used alone in SK-N-SH cells. On the contrary, the combination data were not significantly different from $O_{2/3}$ treatment alone in SK-N-DZ cells, thus suggesting that the obtained changes in cell growth inhibition were due to the effect of $O_{2/3}$ alone.

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XRCC1 INVOLVEMENT IN CELL CYCLE CONTROL AND DNA STRAND BREAK REPAIR: CHARACTERIZATION OF CHINESE HAMSTER CELL LINES AA8 AND EM9

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XRCC1 protein is essential for viability in mammals and is required for efficient DNA single-strand breaks (SSB)

repair. Recently its involvement in DNA double-strand breaks (DSB) rejoining and maintenance of genomic stability has been proposed. Here, we have analysed the biological responses induced by X-ray treatment in two hamster cell lines, AA9 and EM9, expressing the second a truncated polypeptide lacking two-thirds of the normal XRCC1 sequence. In particular we used the alkaline Comet assay to measure the repair kinetics of DNA strand breaks, and the analysis of Chromosomal Aberrations and the expression of phosphorylated histone H2AX to evaluate DSB induction. Furthermore the study of cell cycle modulation has been conducted through flow cytofluorimetric analysis of BrdUrd incorporation.

We found that the repair defective EM9 cells show an higher level of both DSB and SSB and a slower repair kinetic following exposure to radiation. As far as cell cycle progression, both cell lines show a delay in $G_2$ phase at early times after irradiation; at later times a delay during S phase progression is observed only in EM9 cells. These data suggest an overall important role for the interaction of XRCC1 protein with Pol. In XRCC1 deficient cells, the absence of XRCC1/Pol, interaction could compromise not only the efficiency of repair but also perturb replication and progression through S phase after DNA damage.

## CELL GROWTH INHIBITION AND CELL CYCLE PERTURBATIONS INDUCED BY A NEW SYNTHETIC IMINOQUINONE, 5H-PYRIDOPHENOXAZIN-5-ONE, IN HUMAN BREAST CARCINOMA CELL LINES

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5H-Pyridophenoxazin-5-one (PPH) is a new synthetic iminoquinone which has showed to be a potent cytotoxic agent on different cancer cell lines. PPH effects depend by the intercalation into the DNA double strand at the middle 5′-GC-3′ base pairs of the octamer [d(GAAGCTTC)$_2$] and the production, under bio reductive conditions, of free oxygen radicals. The aim of the study was to analyse cell growth and cell cycle kinetics of MCF-7 (p53 wt) and MDA-MB-468 (p53 mut) human breast cancer cell lines after PPH exposure. Treatments were performed with different PPH concentrations (ranging from 0.5 μM up 8 μM) as pulsed (60 min/exposure) or continuous exposure. MCF-7 cells were more sensitive to pulsed treatment than MDA-MB-468 cell line. Cell cycle analysis by PI staining showed a $G_2$M perturbation that was overcome after 72 hours in both cell lines. As expected, continuous treatment caused a strongest cell-growth inhibition and cell cycle perturbation in both cell lines. However, DNA-flow cytometry showed that MCF-7 cells cytotoxicity correlate with an accumulation of cells in the S phase, whereas an irreversible $G_2$M block accompanied MDA-MB-468 cytotoxicity.

## CONFORMATIONAL MUTANT p53 ISOFORM: A NOVEL ALZHEIMER'S DISEASE MARKER?

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Background: Alzheimer's disease (AD) cannot be diagnosed until dementia appears, thus the detection of early disease-related biomarkers is crucial to facilitate the development of new diagnostic tools and drug therapies. Candidate biochemical markers for AD should be molecules representing some of the cerebral pathogenetic processes typical of AD. Alternatively they should represent altered metabolic or cellular processes in the brain or in peripheral tissues from affected patients. In research of secondary markers we recently found an intriguing correlation between p53 and AD. In particular, we demonstrated that fibroblasts from sporadic AD patients specifically express an unfolded and detectable conformational state of p53 that allows to differentiate them from fibroblasts of age-matched non-AD subjects (Uberti et al., 2006). Methods: In this study, we used a rapid and easy flowcytometric approach to investigate the different expression of conformationally altered p53 between AD and non-AD subjects on peripheral blood cells, which represent a more accessible cellular model than fibroblasts. We enrolled more than 150 subjects and tested the content of unfolded p53 in AD, non AD and subjects affected by other dementia. Results: We found that peripheral blood cells from AD specifically expressed increased levels of unfolded p53 in comparison with non AD subjects. A statistically significant correlation was observed when the expression of unfolded p53 and the age of both control subjects and AD patients were considered thus demonstrating that altered p53 is an age-dependent factor. In order to evaluate the diagnostic performance of unfolded p53 as an AD trait marker, we calculated sensitivity and specificity within different age intervals and we found that these values were more significant in subjects up to 70 years of age compared to the values in individuals older than 70 years. Within this specific age interval ($\leq$70 years), sensitivity and specificity were respectively 90% and 77%. Conclusions: The observation of an altered p53 content in young patients is of great importance and could suggest the usefulness of this method especially for younger people, thus supporting its putative application for subjects with mild cognitive impairment (MCI) and with early onset of AD.

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FRACTAL / GLCM STATISTICS CHARACTERIZATION OF EARLY APOPTOSIS IN HUMAN BREAST CANCER CELLS

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An analytical strategy combining fractal geometry and grey-level co-occurrence matrix statistics (GLCM) was devised to investigate ultrastructural changes in oestrogen-insensitive Sk-BR3 human breast cancer cells undergoing apoptosis induced in vitro by 1 ÌM calcimycin. Cells entered the early stage of apoptosis within 24 h of treatment with calcimycin, which induced detectable ultrastructural changes in the plasma membrane and in the chromatin texture of nuclear components as indicated by a general reduction of fractal dimensions and by increased values of most GLCM parameters. Hence, both fractal and GLCM analyses confirmed that the morphological reorganization is imputable to a loss of structural complexity, occurs in the early stage of apoptosis and precedes the onset of conventional cellular markers, which can only be detected during the active phases of the apoptotic process.

IN VITRO AND IN VIVO ANTITUMOUR ACTIVITY OF PYRIMETHAMINE IN HUMAN METASTATIC MELANOMA CELLS

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Pyrimethamine (pyr), a folic acid antagonist, may exert, in addition to anti-protozoan effects, immunomodulating activities including induction of apoptosis in human lymphocytes from lymphoproliferative disorders. Despite these findings recently detected in our laboratory, no data characterizing pyr anticancer activities are available so far. The aim of the present study was to evaluate the in vitro and in vivo antitumor activity exerted by pyr in human metastatic melanoma cells.

Flow cytometric analysis was used to study: i) apoptosis by double staining with annexin V/propidium iodide (PI); ii) the surface phenotype (e.g. CD95 expression); iii) caspase and cathepsin expression and function; iv) mitochondrial membrane potential by using the mitochondrion-selective probe tetramethylrhodamine methyl ester; v) cell cycle distribution by 5-bromo-2′-deoxyuridine (BrdUrd)/ anti-BrdUrd monoclonal antibody and PI. Western Blot analysis was also performed to analyze caspase- and cathepsin-cascade.

Our results indicate that pyr, used at pharmacological concentrations comparable to those used in the clinical practice, induced apoptosis in metastatic melanoma cells via the activation of either cathepsin B and/or caspase-cascade (i.e. caspases 8 and 10) and subsequent mitochondrial depolarization. This seems to occur independently from CD95/Fas engagement. Moreover, pyr induced a marked inhibition of cell growth and a S-phase cell cycle arrest.

In conclusion, our data suggest a mechanism for pyr-mediated apoptosis that bypasses CD95/Fas engagement but overlaps its subcellular pathway. On these bases, pyr is an interesting candidate as pro-apoptotic agent for the treatment of metastatic melanoma.

INHIBITION OF CELL CYCLE AND APOPTOSIS IN HUMAN CERVIX CARCINOMA CELL LINES INDUCED BY RHEIN, QUERCETIN AND PLUMBAGIN AND IN SILICO DESIGNED SEMISYNTHETIC ANALOGS

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Increasing epidemiological and experimental evidences indicate that natural polyphenols are interesting substances in chemoprevention, a new approach to develop efficient strategies of controlling cancer (1). We studied the effect of rhein (antraquinone), quercetin (flavonoid) and plumbagin (naphtoquinone) on wild type (A431) human cervix carcinoma cells line and on cis-platin (CDDP) resistant variant (A431Pt). Cells were treated for 24 hours with (0.1μM–0.1mM) of different natural phenols and cytotoxicity and cell cycle were measured. Results demonstrate that all substances were cytotoxic at micromolar concentration, also causing evident alterations of cell cycle phases and apoptosis. In order to test the likely mechanism of inhibition of cell cycle by natural phenols an hightroughput consensus docking study with ATP-binding pocket of cdk2 protein kinase was performed. Results evidence that some small derivatives of antraquinones, flavonoids and naphtoquinones have likely improved affinity for ATP-binding cleft of cdk2. These derivatives were synthetized and their effects at concentrations (0.1μM–0.1mM) were assayed on cell viability and cell cycle in cervix carcinoma cell lines. Results show that semisynthetic naphtaquinones are more potent cytotoxic compounds than antraquinones and flavonoid analogs, causing cell cycle alterations and apoptosis. Data support natural phenols as chemosensitizing cytotoxic substances (2) and also that in silico design of new phenols derivatives is an useful approach to obtain more selective targeted compounds in cancer therapy.

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DELAYED ENTRY TO QUIESCENCE IN $p21^{CDKN1A}$-NULL HUMAN FIBROBLASTS: A POSSIBLE INVOL-VEMENT OF DNA DAMAGE CHECKPOINT

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The cyclin-dependent kinase inhibitor $p21^{CDKN1A}$ is involved in several cellular processes including cell cycle

arrest induced by DNA damage, cell differentiation and senescence. In contrast, its role in cell cycle exit to quiescence (G0) is more controversial. Here, we have investigated the role of p21 in the quiescent growth arrest of human fibroblasts induced by contact inhibition and mitogen withdrawal. The results have shown a delayed entry in G0 phase by $p21^{-/-}$ fibroblasts, as compared with parental $p21^{+/+}$ cells. This effect was concomitant with a significant increase in p21 and p27 protein levels only in $p21^{+/+}$ cells, whereas p16 and p19 protein levels were not modified in both cell lines. Analysis of cell cycle-related gene expression by microarray revealed an increased expression of proteins implicated in cell cycle checkpoints, only in $p21^{-/-}$ cells. The phosphorylation status of histone $H_2AX$ and Chk1 suggested an activation of DNA damage checkpoint in p21-null cells driven to quiescence. RNA interference-mediated suppression of p21 in HT-1080 tumour cells determined efficiently a delay in cell growth inhibition after serum starvation. Experiments are in progress to determine the phosphorylation status of histone H2AX and Chk1. Finally, Comet assay combined with pulse-labelling of replicating DNA with BrdU indicated a weak nuclear fragmentation in $p21^{-/-}$ cells, suggesting that loss of p21 facilitated the occurrence of DNA replication stress.

## DNA DAMAGE REPAIR, CHECKPOINT ACTIVATION AND APOPTOSIS IN CORD BLOOD STEM CELLS

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Hematopoietic stem cells have the capacity to self-renew and to undergo a process of commitment to multipotential progenitors, which in turn give rise to mature blood cells. Because of their special function it is axiomatic that the genome of stem cells must be maintained free of mutations to minimize the risk of propagating mutations. To better understanding the molecular mechanisms of controlling genomic stability in stem cells we performed a comparison of the DNA repair capacity of ionizing radiation-induced DNA damage in cycling hematopoietic stem cells (HSCs, $CD34^+$) *vs* more differentiated cells (late precursors, $CD34^-$). We studied the ability of stem cells to repair γ-radiation induced double strand breaks (DSBs) and to produce chromosome damage by analysing phosphorylated histone H2AX and micronuclei in binucleated cells. Though the induction of DSBs was similar in HSCs and late precursors, the kinetics of DSBs repair were faster in the former. This was accompanied by a decreased frequency of micronuclei in HSCs. Checkpoint activation was also evaluated in the two cell population by FACS analysis after γ-irradiation. Late precursor showed an efficient block in cell cycle progression both at G1/S and G2/M. A less efficient G1/S block was observed in HSCs, although the G2/M arrest was maintained. HSCs were more sensitive in comparison to late precursors to γ-radiation-induced apoptosis measured by annexin V. In conclusion, HSCs accumulate less chromosomal damage than late precursor, probably because of a more pronounced apoptotic response.

## EFFECTS OF 17β-ESTRADIOL AND SELECTIVE ESTROGEN RECEPTOR MODULATORS ON PROLIFERATION, DEATH AND DIFFERENTIATION OF HUMAN OSTEOBLAST-LIKE CELLS

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To investigate the pathophysiology of osteoporosis, we evaluated the relevance of the action of the estrogen receptor (ER) α and ERβ in mediating the proliferative, apoptotic and differentiative effects induced by 17β-estradiol ($E_2$), natural phytoestrogens genistein (G) and daidzein (D), soy extract and synthetic 4-hydroxytamoxifen (4OH-T), in the human osteoblast-like U2OS cell line stably expressing ERα or ERβ.

$E_2$, G, D and soy extract, in both cell lines, decreased cell proliferation up to 75%, and inhibited cell cycle progression markedly reducing the number of cells entering the S phase. Treatments also promoted the activation of the apoptotic program and changes in cell morphology typical of osteoblast differentiation. On the contrary, 4OH-T exerted weak estrogenic effects by ERα, but acted as an antagonist by ERβ.

These findings show that $E_2$ and phytoestrogens promote ER-mediated osteoblast maturation and, therefore, maintenance of bone mass, suggesting potential targets in the treatment of bone diseases.

## ANTIPROLIFERATIVE ACTIVITY OF THE NEW RESVERATROL-DERIVATIVE 4, 4′-DIHYDROXY-STILBENE

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The synthesis of resveratrol derivatives allowed to identify in the 4′-hydroxystyryl moiety the structural determinant required for its antiproliferative activity. In this study we have evaluated the properties of a new derivative 4,4′-hydroxyl-stilbene, which has two hydroxystyryl components. This compound showed a higher antiproliferative effect, compared to resveratrol. In fact, a 50% inhibition of cell growth was induced by the new compound at a concentration 4-fold lower than resveratrol. In addition, increasing concentration of resveratrol determined a progressive accumulation of cells at the beginning of S phase, whereas the new compound, at equimolar concentration, arrested cells in G1 phase. DNA replication assays in vitro showed a higher activity of resveratrol in inhibiting DNA synthesis. Analysis of cell cycle-related gene expression by microarray revealed that both compounds altered the expression profile observed in control samples, with an increased expression of G1/S-phase cyclins, CDK inhibitors, and proteins implicated in cell cycle checkpoints. Analysis at the protein levels of cyclin D and E, $p21^{CDKN1A}$ and $p27^{CDKN1B}$, p53,

chromatin-bound form of MCM2 and PCNA, together with the phosphorylation status of retinoblastoma protein, suggested that 4,4′-hydroxyl-stilbene affects additional targets than resveratrol, thus explaining the stronger antiproliferative properties of this new analogue.

## RELATIONSHIP BETWEEN CELL CYCLE EFFECTS AND MICROTUBULAR ALTERATIONS DURING CELL DEATH INDUCED BY ANTIMICROTUBULAR AGENTS IN NIH/3T3 SUBCULTURES

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We studied Taxol (TAX) and Vinblastine (VBL) effects on cell cycle and microtubules of two NIH/3T3 subcultures with low (NIHb) and high (NIHs) proliferative activity. By morphocytometric and ultrastructural techniques, variable responses to the drugs were observed: in NIHb cells, VBL induced major microtubule depolymerization, prevalence of tubulin paracrystals and intense micronucleation; in NIHs cells, VBL caused low depolymerization and macrotubule aggregates, with lower micronucleation and apoptosis increase. DNA static cytofluorometry of cells with paracrystals or macrotubules permitted to correlate the appearance of these tubulin aggregation forms with the cell cycle phases. In NIHb line, the DNA content curves, in cells with paracrystals or macrotubules, showed a similar trend, with a higher frequency of both anomalies in the G2/M phase. In NIHs line, paracrystals and macrotubules are found in G2/M cells, while G1 cells showed prevailingly paracrystals. TAX induced the appearance of microtubule bundles in both cell lines. Nevertheless, the prevalence of circular bundles was found in NIHb cells, while a higher number of linear bundles was shown in NIHs cells. In NIHb cells, circular bundles were related to a higher induction of canonic and alternative apoptosis. DNA content analysis, in TAX-treated cells with linear or circular microtubule bundles, showed that the latter were present with high frequency in NIHb cells in all the cell cycle phases; in NIHs cells, they appeared, with low frequency, prevailingly in the G2/M phase. Furthermore, in NIHs cells, the appearance of linear microtubule bundles involved G1 cells, and could be related to a lower presence of micronuclei. These finding showed that microtubule reorganization could play a role in the progression of nuclear fragmentation/micronucleation relating to cell death.

## DYNAMICS OF CELL CYCLE PHASE PERTURBATIONS BY TRABECTEDIN (ET-743) IN NER DEFICIENT AND PROFICIENT CELLS, UNRAVELLED BY A NOVEL MATHEMATICAL SIMULATION APPROACH

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Trabectedin (ET-743, Yondelis®) is a natural marine product with antitumour activity currently in phase II/III clinical trials. Previous studies have shown that cells hypersensitive to UV-rays because of "Nucleotide Excision Repair" (NER) deficiency were resistant to trabectedin. The purpose of this study was to investigate whether this resistance was associated with different drug-induced cell cycle perturbations. An isogenic NER-proficient cellular system (CHO AA8) and a NER-deficient one (CHO UV-96), lacking a functional ERCC-1 were studied.

Flow cytometric assays showed a progressive accumulation of cells in $G_2M$ in NER-proficient but not in NER-deficient cells. Applying a computer simulation method, we realized that the dynamics of the cell cycle perturbations in all phases were complex. Cells exposed to trabectedin during $G_1$ and $G_2M$ first experienced a $G_1$ block, while those exposed in S were delayed in S and $G_2M$ but eventually divided. In the presence of functional NER, exit from the $G_1$ block was faster, then cells progressed slowly through S phase and were subsequently blocked in $G_2M$ phase. This $G_2M$-processing of trabectedin-induced damage in NER-proficient cells was unable to restore cell cycling, suggesting a difficulty in repairing the damage. This might be due either to the fact that important damage was left unrepaired by previous $G_1$ repair, or that NER activity itself caused DNA damage, or both. We speculate that in UV-96 cells repair mechanisms other than NER are activated both in $G_1$ and $G_2M$.

## BYPASS OF METHYLATION DNA DAMAGE BY TRANSLESION POLYMERASES AND ITS ROLE IN TOXICITY

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The ability of Y-family DNA polymerases to bybass damaged bases has been extensively investigated in vitro, but little is known on their biological role in mammalian cells. Silencing of DNA Pols κ was achieved by siRNA in human cell lines (<20% residual protein). Knock-down of Pol κ sensitized both HeLa and A2780 cells to killing by N-methyl-N-nitrosourea (MNU). This increased toxicity was associated with the presence of $O^6$-methylguanine ($O^6$-meGua) in DNA since a) sensitization was observed only in the presence of the MGMT inhibitor $O^6$-benzylgua-nine and b) modulation of killing by MMS was less pronounced. Silencing of Pol κ in HeLa cells resulted however in a modest trend of increased MNU-induced mutagenesis. The absence of Pol κ lead to an increased fraction of HeLa cells with RAD 51 foci after MNU treatment. This suggest that bypass of $O^6$-meGua by this Pol might control the level of homologus recombination associated with persisting methylation damage. The cytofluorimetric analysis of cell cycle progression after MNU treatment showed no clear difference in Pol κ-depleted cells compared to untransfected cells; the absence of a clear delay in the transition of MNU-treated cells through the S phase was confirmed in HeLa cells pulsed with Brd, harvested at various times after MNU treatment and analysed by FACS. In addition no differential increase in formation of γ-H2AX foci, a marker of double strand break at collapsed replication forks, was observed in Pol κ silenced cells. This suggests that activation of homol-

ogous recombination pathways to process $O^6$-meGua is not necessarily associated with a collapse of replication forks.

EXPRESSION OF APOPTOSIS MARKERS ON UMBILICAL CORD BLOOD (UCB) CD34+ CELLS AFTER 10% AND 5% DIMETHYLSULFOXIDE (DMSO) CRYOPRESERVATION

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UCB is being used as a source of hematopoietic stem cells for the bone marrow reconstitution and commonly is cryopreserved with 10% DMSO. The aim of our study was to evaluate the viability and apoptosis by flow cytometry on UCB CD34+ cells comparing 10% and 5% DMSO cryopreservation. After red blood cell depletion and positive selection of CD34+ cells, UCB samples (n = 8) were divided in equal volumes and cryopreserved either with 10% or 5% DMSO. After thawing and removal of DMSO, apoptosis was evaluated using the following markers: Apo2.7, Bcl-2, CD95, Annexin V and 7AAD. Each apoptosis marker expression was calculated as the ratio between the mean fluorescence intensity (MFI) of positive cells and MFI of the isotypic control.

| DMSO | UCB | Apo2.7/ Bcl2 (MFI) | CD95 (MFI) | Annexin V | (MFI)7AAD+ (%) |
|---|---|---|---|---|---|
| 10% | n = 8 | 1.66 ± 1.02 | 1.31 ± 0.49 | 1.27 ± 0.79 | 0.12 ± 0.14 |
| 5% | n = 8 | 1.54 ± 0.39 | 1.25 ± 0.47 | 0.92 ± 0.26 | 0.06 ± 0.06 |

No significant difference was observed between the two DMSO concentrations in term of apoptosis. In conclusion, 5% DMSO concentration can be routinely used in UCB banks because expression of apoptosis markers on CD34+ cells was not increased in comparison with 10%. Concentration of 5% DMSO could allow us a minor infusion-related toxicity especially in pediatric patients.

## CELL THERAPY

CIRCULATING ENDOTHELIAL CELLS IN ADVANCED COLORECTAL CANCER PATIENTS TREATED WITH BEVACIZUMAB-BASED COMBINATION THERAPY: A FLOW CYTOMETRIC STUDY

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Background: The flow cytometric (FCM) evaluation of circulating endothelial cells (CECs) and their progenitors (CEPs) has been proposed as a surrogate biological marker of angiogenesis because the well-known correlation with tumor angiogenetic activity and growth. CECs and CEPs blood concentrations in untreated cancer pts are significantly increased in comparison to healthy subjects, correlating with the tumor progression. Recent data suggest that their modification during antiangiogenetic therapy, a promising approach to cancer treatment, have a potential role as prognostic markers in breast cancer pts. No data are available on the effect of antiangiogenetic Bevacizumab-based first-line therapy on blood concentration of distinct population of CECs and CEPs in metastatic colorectal cancer (mCRC). Material and methods: We analyzed blood levels of CECs (resting and activated) and CEPs by a 4-colour FCM in 15 normal donors (M/F: 10/5, median age 37 yrs), in 5 mCRC pts treated with first-line chemotherapy (CT) (M/F: 1/4, median age 67 yrs) and in 8 mCRC pts receiving a fist-line therapy including Bevacizumab (M/F : 4/4, median age 56 yrs). Resting CECs were defined as negative for CD45 and CD106 and positive for CD34 and CD146. Activated CECs were defined as CD45−, CD34+, CD146+ and CD 106+ cell. CEPs were depicted by the expression of the stem cell marker CD133. Results: With respect to normal donors, mCRC pts treated with CT alone in first-line setting show a decrease of absolute number of the two CEC subsets and of the CEPs. At the same time, Bevacizumab-based therapy correlates with a trend toward the increase of CEPs and CECs, especially in activated subsets, in comparison to mCRC pts treated with CT alone. Conclusions: The determination of CECs and CEPs by FCM is a rapid and effective method for monitoring the clinical impact of antiangiogenetic therapies in mCRC pts.

MDL 72527, A LYSOSOMOTROPIC COMPOUND, ENHANCES THE CYTOTOXIC EFFECT OF THE SPERMINE OXIDATION PRODUCTS ON MULTIDRUG RESISTANT MELANOMA CELLS

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In situ formation of cytotoxic products by an enzyme-catalyzed reaction is a recent approach in anticancer therapy to overcome multidrug resistance to cytotoxic agents. Cell survival experiments demonstrated that resistant human melanoma cells (M14 ADR) were more sensitive than the corresponding wild type cells (M14 WT) to the hydrogen peroxide and aldehydes, the products of bovine serum amine oxidase (BSAO)-catalyzed oxidation of spermine. Pretreatment with the polyamine oxidase inhibitor $N,^1N^4$-bis (2,3-butadienyl)-1,4-butanediamine (MDL 72527), a lysosomotropic compound, sensitized cells to toxic spermine metabolites. The combined effect caused by spermine oxidation products and MDL 72527 was particularly evident in multidrug resistant cells. Observations by transmission electron microscopy revealed the formation of numerous cytoplasmic vacuoles and lysosomes after MDL 72527 treatment, whereas the combination MDL 72527 and BSAO/spermine induced evident mitochondrial damage.

Flow cytometric and confocal microscopic analyses of cells stained with acridine orange, suggested that the lysosomotropic effect of MDL 72527 is the principal cause of its sensitizing action. Since it is known that the release of lysosomal enzymes produces oxidative stress and apoptosis, we evaluated intracellular ROS content by DHR123 staining and apoptotic cell death by AnnexinV-FITC labelling. The results confirmed that enzymatically produced cytotoxic agents in association with the polyamine oxidase inactivator MDL 72527 activate the cellular mechanisms leading to cell death.

These findings suggest that lysosomotropic compounds, such as MDL 72527, used alone or in association, might be promising anticancer agents, mainly against multidrug resistant tumor cells.

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PAX5/TEL ACTS AS A TRANSCRIPTIONAL REPRESSOR, CAUSING DOWN MODULATION OF CD19 AND RESISTANCE TO TGFbeta1 ANTI-PROLIFERATIVE AND PRO-APOPTOTIC EFFECTS IN preBI CELLS

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*PAX5* is a transcription factor essential for B-cell development. Recently, it has been found as frequent target of aberrancies in childhood ALL (30% of B-cell ALL cases), showing monoallelic loss, point mutations or chromosomal translocations. The role of these aberrancies is still poorly understood. Our group first described the *PAX5/TEL* chimeric gene, from the translocation t(9;12) in an ALL patient. Preliminary results showed the nuclear localization of PAX5/TEL, which recruited mSin3A corepressor. Aim of the study was to investigate the functional roles of PAX5/TEL protein in vitro in murine wild type preBI cells, isolated from mouse fetal liver by sorting as being B220+/C-KIT+/CD19+. The experiments have been performed both in a heterogeneous preBI cell population and in a single clone, isolated by single-cell sorting. PAX5/TEL preBI cells showed down modulation of CD19 and B220 antigens. RQ-PCR analysis showed down-regulation of *BLNK* and *MB1* expression. $PAX5^{-/-}$ preBI cells transduced with PAX5/TEL did not show any difference with the parental cells. In a transwell assay, PAX5/TEL preBI cells showed increased migration towards CXCL12 and the up-regulation of *CXCR4* receptor level. After IL7 starvation, PAX5/TEL preBI cells showed a survival advantage without acquiring a long term cytokine independence. In absence of IL7, preBI cells completed the rearrangement of the μ heavy chain at a cytoplasmic level, but PAX5/TEL cells showed an impairment in its expression. PAX5/TEL cells were resistant to TGFbeta1 anti-proliferative and pro-apoptotic effects, continuing to proliferate. In conclusion, PAX5/TEL protein functions as an aberrant transcription factor with repressor function, interfering with the processes of B-cell differentiation and migration, inducing resistance to apoptosis.

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ANTITUMOR CELLULAR IMMUNORESPONSE IN ADVANCED COLORECTAL CANCER PATIENTS TREATED WITH ANTIANGIOGENETIC THERAPY

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Background: Vascular endothelial growth factor (VEGF) is a key agent in promoting and sustaining the immunetolerance during the cancer growth, particularly because of the indirect impairment on the functional maturation of dendritic cells (DCs). The block of VEGF in preclinical murine models enhances the efficacy of cancer immunotherapy in colorectal carcinoma. Bevacizumab, an humanized monoclonal antibody against VEGF, is largely employed in the treatment of metastatic colorectal cancer (mCRC) pts in addition to chemotherapy (CT), and its in vivo impact on pts immune system has not been clarified. Material and methods: We have studied the impact on immunosystem of first-line Bevacizumab-based combination therapy in 27 pts with mCRC (M/F: 20/7, median age: 55 yrs), in absence of clinically relevant infections. Data were compared with 21 mCRC pts who have received CT alone as first-line treatment (M/F: 16/5, median age: 56 yrs) and with 40 healthy subjects (M/F: 20/20, median age: 40 yrs). The immunological profile of our pts was evaluated by flow cytometric analysis of different PB lymphocyte and DC subsets. Results: With respect to normal donors, a significant decrease of absolute lymphocyte number, CD4 T lymphocytes, CD19 and CD20 B-lymphocytes, NK cells and DCs was evidenced in mCRC pts treated with CT alone. Bevacizumab addition to CT didn't affect the B lymphocyte and the NK compartments, but statistically significant increased CD4 T lymphocytes ($p < 0.003$). At the same time, Bevacizumab administration was associated with a significant increase of absolute DC number and of their cellular subset ($p < 0.001$), with a decrease of DC humoral subset ($p < 0.002$). Conclusions: The in vivo T-cell mediated response seems to be improved by first-line Bevacizumab-based therapy in mCRC pts. This data suggests that the VEGF blockade could have a synergistic effect in cancer immunotherapy programs for mCRC pts.

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THE PLANT ALKALOID VOACAMINE INDUCES AUTOPHAGY AND CHEMOSENSITIZING EFFECT ON MULTIDRUG RESISTANT TUMOR CELLS

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Multidrug resistance (MDR) phenotype in tumor cells is an adverse factor for the efficacy of chemotherapeutic regimens. MDR is generally associated with reduced intra-

cellular drug accumulation and changed distribution, due to overexpression of transport proteins, such as P-glycoprotein (Pgp). In the present study, the possible chemosensitizing effect of voacamine (VOA), an alkaloid extracted from *Peschiera fuchsiaefolia,* was evaluated on both-sensitive and drug-resistant cell lines. In MDR osteosarcoma cells, the pretreatment with VOA induced a significant increase of doxorubicin (DOX) retention, that became comparable to that observed in the parental sensitive counterparts, accompanied by a noticeable increase of the cytotoxic effect. To verify if VOA is a substrate of Pgp, we evaluated by flow cytometry and confocal microscopy the reactivity modulation of the anti-Pgp MAb UIC2, which recognizes an epitope of the drug transporter in its functional conformation. The results obtained indicated that the increased cytotoxic effect of DOX on resistant cells was due to a competitive action of the plant extract against P-gp function. To evaluate if the enhancement of the cytotoxic effect was associated with apoptosis induction, cell cycle analysis and DAPI staining were carried out. These methods did not reveal evident apoptotic cell death. Moreover, flow cytometric analysis performed after double labeling with Annexin V-FITC and Trypan blue showed a very low rate of apoptosis in the resistant cell line treated with the combination VOA+DOX. Interestingly, electron microscopy observations showed that most of these cells were in the stage of autophagic process, containing a large number of vacuolar autophagosomes.

Even though the role of autophagy in tumor progression and in the response of cancer cells to antitumoral drugs has not been established at all, the possibility of affecting the efficacy of anticancer therapies by using substances capable of inducing autophagy and, possibly, with limited undesirable side effects, might represent a promising innovative strategy.

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## A NEW TRANSFECTION STRATEGY IN THE PHOTODYNAMIC THERAPY OF MALIGNANT GLIOMAS

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Malignant gliomas represent the most common primary brain tumor: more than 50% of them are glioblastoma multiforme (GBM). Photodynamic therapy may offer a very good chance of targeted destruction of infiltrating GBM cells, thus increasing the survival time and recurrence-free interval of GBM patients. Among photosensitizing agents, meta-tetrahydroxyphenylchlorin (*m*-THPC) is promising for the treatment of brain tumors. In this study we investigated the transfection activity of dimyristoyl-*sn-glycero*-phosphatidylcholine (DMPC) liposomes, containing a cationic gemini surfactant, loaded with *m*-THPC on human and murine glioblastoma cell lines. The uptake (flow cytometry) and the intracellular distribution (confocal microscopy) of *m*-THPC, loaded in several formulations of cationic liposomes, were analyzed by making a comparison with those obtained by employing the same chlorin in the pharmaceutical form (Foscan®). Moreover, by cloning efficiency assay the potential therapeutic efficiency of chlorin delivered by liposome formulations was compared to that of the pharmaceutical compound, before and after irradiation with laser light at 652 nm.

The obtained results indicated that cationic liposomes (i) transferred *m*-THPC in glioblastoma cells more efficiently than pharmaceutical formulation; (ii) significantly ($p << 0.001$) increased the *m*-THPC cytotoxic effect after laser irradiation; (iii) seemed to exert their cytotoxic action in the early phase of interaction with the cells, during adhesion to the plasma membrane.

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## EFFECTS OF PEGFILGRASTIM ADMINISTRATION ON NEUTROPHIL APOPTOSIS IN BREAST CANCER PATIENTS TREATED WITH DOSE-DENSE CHEMOTHERAPY REGIMENS

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Pegfilgrastim is a covalent conjugate of filgrastim and polyethylene glycol with an increased elimination half-life. At our center two clinical trials are currently ongoing that will further evaluate pegfilgrastim utilized in dose-dense regimens for breast cancer patients both as neoadjuvant and adjuvant approach. Twenty patients were enrolled in two different multicenter clinical trials. Four patients received 4 courses of concomitant Anthracycline and Taxane chemotherapy as primary systemic treatment and 16 patients received 8 courses of Anthracycline and Taxane on sequential scheme, both every 2 weeks (dose dense schedule), with pegfilgrastim administered from 24 to 72 hours after each chemotherapy course. On peripheral blood buffy coat smears obtained before starting treatment and before each chemotherapy course we analyzed the following parameters in neutrophils: apoptosis by TUNEL technique, actin polymerization using phalloidin labeled with FITC, and alkaline phosphatase activity by cytochemistry. Our aim was to evaluate the influence of pegfilgrastim on these biological features. After stimulation with pegfilgrastim we observed: stability of the absolute neutrophil count for the whole duration of treatment and no infectious events; a reduction of neutrophil apoptosis rate in comparison with control patients treated with standard chemotherapy courses without filgrastim support; persistent abnormalities of actin assembly in neutrophils, indicative of changes in cytoskeleton organization; a significant increase of the leucocyte alkaline phosphatase activity, that is a sensitive marker of myeloid differentiation. These results suggest that pegfilgrastim may improve the neutrophil function in patients with cancer exposed to chemotherapy by inhibiting the accelerated apoptosis and prolonging survival. This effect may, at least in part, be dependent on the influence of pegfilgrastim on actin cytoskeleton organization.

## ENVIROMENTAL SCIENCES AND TOXICOLOGY

### FLOW CYTOMETRY CHARACTERIZATION IN CITRUS INTERPLOID CROSSES

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One of the most important goals in *Citrus* genetic improvement is to obtain seedless cultivars. Interploid crosses (2X×4X) among suitable Citrus variety and somatic hybrids were carried out to obtain seedless triploid plants. Three different allotetraploid somatic hybrids (2n = 4X) were used as pollen donors in sexual crosses with a diploid 'Femminello' Italian lemon; autotetraploid 'Dancy' mandarin (2n = 4X) was used as a pollen parent for crossing with diploid (2n = 2X) grapefruit and mandarin cultivars.

The ploidy level of interploid crosses was estimated by flow cytometry comparing DNA relative fluorescence intensity from DAPI stained nuclei to an internal DNA standard.

Citrus nuclei were released from leaves after chopping according to our standard procedure. Flow cytometry analysis and DNA histogram quality were both affected by the different amount of intracellular oil vesicles generated by each crosses. Nevertheless, DAPI staining strong fluorescence allowed us for a clear ploidy discrimination in respect to PI which showed DNA histograms with higher CVs. Surprisingly, we found tetraploid plants out of diploids and triploids offsprings after 2X×4X crosses, possibly generated by induced polyploidization after pollen stimulation of the egg cell.

### DIFFERENTIAL EFFECT OF POLYUNSATURATED ALDEHYDES PRODUCED BY DIATOMS ON NATURAL BACTERIAL COMMUNITIES

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Diatoms produce polyunsaturated aldehydes which are teratogenous for the copepods feeding on them. These secondary metabolites are also able to reduce other phytoplankton growth by inhibiting cell division and inducing death by apoptosis. We have tested three of these aldehydes on natural communities of marine bacteria from a coastal area of the Mediterranean Sea during incubation experiments. Cell concentrations, respiration and bacterial production were reduced in samples inoculated with the three aldehydes or with a mixture of them. However, it is possible that only part of the community was responsible for the drop in average values. To test this hypothesis, microautoradiography has been used coupled with FISH using bacterial group-specific probes to investigate the occurrence of differential toxicity of these aldehydes on different bacterial groups. The preliminary results show that some bacterial groups bacteria were affected by these compounds in terms of slower activity, while others were not affected at all when compared to the controls. The results suggest that during blooms of aldehyde-releasing diatoms, some bacterial group may profit of the toxic effect on growth to outgrow competitors for the same resources and dominate at times. These bacteria may also possess resistance or detoxification mechanisms against these compounds, probably maintained by their closed association with diatoms.

### MOLECULAR ANALYSIS AND FLOW CYTOMETRY EVALUATION OF NUCLEAR DNA IN *CITRUS LIMONIMEDICA* LUSH. TO ASSESS GENETIC VARIABILITY AND TO TRACE PHYLOGENETIC RELATIONSHIP AMONG LEMON AND CEDAR

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In 1910, Lushington first described grapefruit (*Citrus limonimedica* Lush.) as a species, but his observations were performmed on domesticated plants only. *Citrus limonimedica* is a cultived species in India, Cina and Japan. In Italy, few varieties of this plant are known (*C. limonimedica* 'Bicolor', 'Canarone', 'Florentina', 'Maxima', 'Paradisi', 'Perettone', 'Pigmentata', 'Rubra', 'Rugoso', 'Sanctus Dominicus') and they were utilized as ornamental plants since Medici age in Florence. Regarding fruit size and shape, a ramarkable variability exists among italian *Citrus limonimedica* varities. This fact brings to the possibility that italian *Citrus limonimedica* could be spontaneous hybrids among lemon (*Citrus limon* (L.) Burm.) and cedar (*Citrus medica* L).

In our research, we have focused our attemption to characterize the variability between individuals and between putative supposed ancestors to trace back phylogenic development of this variety belonging to *Rutaceae* family, *Aurantioideae* subfamily, genus *Citrus*.

### DIVERSITY OF EPILIMNETIC AND HYPOLIMNETIC BACTERIAL COMMUNITIES IN A DEEP SUBALPINE LAKE

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Large lakes of Europe are undergoing an increase of hypolimnetic temperature as a consequence of the global warming. This will likely determine in deep oligomictic lakes a decrease in frequency of overturn events and in winter mixing depths. The consequent rise in separation of epi and hypolimnetic waters could possibly change the microbial diversity between the two zones. To provide a baseline useful to evaluate the possible future changes we

evaluated the bacterial diversity in epi and hypolimnetic waters of Lago Maggiore, a large deep oligotrophic lake in Northern Italy. We compared abundance, morphology, activity and genetic features of bacteria in the two zones. The average cellular volume of bacteria in epilimnium resulted significantly lower that in hypolimnium (t-test, $P < 0.001$). The mean bacterial carbon production from [$^{14}$C]-Leucine uptake was 0.37 and 0.04 $\mu g\ C\ l^{-1}\ h^{-1}$ in epilimnetic and hypolimnetic waters, respectively. The Denaturating Gradient Gel Electrophoresis (DGGE) profile showed a high genetic diversity between the two zones particularly in late summer. The analysis of samples with CAtalyzed Reporter Deposition- Fluorescence In Situ Hybridization (CARD-FISH) showed a remarkable gradient of increasing abundance of *Archaea* respect to *Eubacteria* along the water column from surface to deep waters.

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FLOW CYTOMETRY (FCM) REVEALS CELLULAR RESPONSES TO STEM CELL FACTOR (SCF) IN MOLLUSCAN IMMUNOCYTES

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During the last decade, FCM has been successfully applied as a powerful tool for investigating the cellular machinery of invertebrates. Analyses have focused on functions linked to innate immunity, such as phagocytosis and natural killer cell activity, as well as on the sensitivity of invertebrate cells to a particular stress or to different chemical agents (Ottaviani and Cossarizza, 1990, Cossarizza, 2005). In bivalve molluscs, immunocytes (haemocytes) resemble in morphology and function the monocyte/macrophage lineage.

Stem Cell Factor (SCF) is a member of mammalian haematopoietic cytokines, a group of glycoproteins that regulate the growth and differentiation of haematopoietic cells and functionally activated mature neutrophils or macrophages. In this work, FCM was utilised to evaluate the possible effects of human recombinant SCF (50 ng/ml) on expression of cellular receptors, cell number and DNA content of molluscan hemocytes. Different haemocyte functional parameters (lysosomal membrane stability-LMS, lysozyme release-LR, phagocytosis) were also measured.

Flow cytometry analysis showed that SCF significantly affected haemocyte number, cell receptors and slightly modify cell cycle; in particular, increases in the number of granular (phagocytic) haemocytes were observed. Moreover, SCF induced significant changes in haemocytes functional parameters, indicating increase in phagocytic activity and reduction in lysosomal membrane fusion processes.

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BACTERIOPLANKTON COMMUNITY COMPOSITION BY CARD-FISH AND FLOW CYTOMETRY IN CENTRAL ADRIATIC SEA

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The knowledge of seawater microbial diversity is important to understand the community structure and pattern of distribution. Flow cytometry and CARD-FISH have been used for the analysis of different types of microorganisms in marine samples and to study the diversity of marine bacterioplankton communities (Pernthaler et al., 2002; Schönhuber et al., 1997; Li et al., 1995).

In this work, bacterial community of sea and estuarine water in two different sampling stations ("Foce Metauro" and "Sassonia") in the Adriatic coast has been compared. In particular, the abundance of total eubacteria, gamma-proteobacteria, alfa-proteobacteria, sar11, beta-proteobacteria, Cytophaga-Flavobacterium and archaeabacteria was evaluated for each sampling station by CARD-FISH while the evaluation of autofluorescence, absolute, viable and actively respiring bacterial cell counts was performed by flow cytometry.

"Force Metauro" and "Sassonia" were characterized by differences in bacterial abundance, cell viability and bacterial composition. Among Eubacteria, the most abundant group in "Sassonia" was alfa-proteobacteria while Cytophaga-Flavobacterium was the most abundant in "Foce Metauro" where beta-proteobacteria reached their maximum values, as they are typical of freshwater environments. A more in deep analysis will be necessary to better describe bacterioplankton community composition and its variability both on spatial and seasonal point of view.

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CARD-FISH IN FLOW: RESULTS AND TROUBLESHOOTING

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The combination of flow cytometry with FISH and amplification techniques is a powerful method for the enumeration of cells in pure cultures and potentially in environmental samples (Sekar et al., 2004; Biegala et al., 2003; Fuchs et al., 2000; Lange et al., 1997; Schonhuber et al., 1997; Wallner et al., 1993). The combination of rapidity and accuracy of FCM together with sensitivity and specificity of oligonucleotide probes allows bacteria counting and identification in different samples.

The main problem with environmental samples is the low ribosomal content of many aquatic bacteria. In these cases, FCM coupled with CARD-FISH gives maximum detection rates of Eubacteria (EUB338I-III labelled bacteria) of about 25% of total cells. In this work, FCM, CARD-FISH and RWCD method were combined and applied on *E. coli* pure culture and river water samples. At the same time, CARD-FISH protocol on 0,22 $\mu$m membrane was performed as a positive control. About 90% of *E. coli* were detectable by FCM after hybridization while the detection of river bacteria was quite lower. The problem was that in some cases only few bacteria hybridized with the oligonucleotide probe and the signal was not very strong, as confirmed by microscopy observation. What's the matter when passing from membrane to suspension? In this study, different attempts were made to improve hybridization efficiency and signal fluorescence intensity.

## RAPID QUANTIFICATION OF FAECAL INDICATORS IN WATER TREATMENT USING FLOW CYTOMETRY

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The aim of the research was the development and the application of flow cytometric methods for the rapid quantification of conventional indicators of faecal contamination in water resources and in water treatment. The methods were developed initially by testing pure strains of E. coli (ATCC 25922), being E. coli well representative of total and faecal coliforms. Bacteria suspensions were diluted in PBS reaching a concentration of 5000–7000 cellule/μL and stained with SYBR-Green I and Propidium Iodide for the assessment of total cells viability. The presence of total coliforms was investigated by using the fluorogenic dye FDG, enzymatically hydrolysable by β-D-galactosidase. The fluorgenic substrate FDGlucU, hydrolysable by the enzyme β-D-glucuronidase, was used for discriminate E. coli. Flow cytometric analyses were performed by comparing a laser flow cytometer (A40, Apogee) and an arc lamp flow cytometer (Bryte-HS, Biorad), using exitation at 488 nm. The optimal procedure for quantification of total coliforms and E. coli was based on: coarse filtration, ethanol addition for cell permeabilisation at 37°C, washing by centrifugation followed by resuspension in PBS, staining of cells with FDG (or FDGlucU). Results of coliform concentrations obtained by flow cytometry were compared to conventional microbiological analysis based on plate counts (APAT-IRSA, 2003) and considered as the reference for the assessement of water quality. Preliminary results obtained from the application of the proposed methods to surface waters were described. In conclusion the following aspects were underlined: (1) specificity of the fluorogenic substrated tested, (2) rapidity and efficacy of the flow cytometric approach in quantifying faecal coliforms in surface waters and along water treatments.

## DETECTION OF OXIDATIVE DNA DAMAGE IN A549 CELLS EXPOSED TO SMOKE EXTRACT FROM COMMERCIAL CIGARETTES

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Cigarette smoke is a complex mixture of chemicals, some of which known carcinogens and mutagens. We evaluated by Fpg comet assay direct-oxidative DNA damage on epithelial lung cells (A549) exposed to cigarette smoke extract (CSE) from three commercial cigarettes (one filterless cigarette (A) and two common brands of filter cigarettes (B and C)). CSE was obtained from each cigarette combusted with a siringe-driven apparatus and dissolved in serum-free RPMI medium. The cells were exposed for 30 min to 1.25, 2.5, 5 and 10% of CSE from a cigarette (A, B or C) and comet percentage and Tail moment from fpg-enzyme treated (TMenz) and untreated (TM) cells were evaluated. As expected the concentrations of CSE constituents: mono-aromatic hydrocarbons, aromatic amines, PAHs, nitroso-amines and metals were higher in filterless cigarettes (A) and a difference for total PAHs, nitroso-amines, aromatic amines, cadmium and benzene was also found between the two brands of filter cigarettes with higher values in cigarette (B). A significant increase of comet percentage was found after exposure to 5% and 10% of CSE (A), while not significant increases were found for (B) and (C) CSE. A significant increase of TMenz and a slight increase of TM were found after exposure to 5 and 10% of CSE (A), indicating an induction of oxidative DNA damage. A mild increase of TMenz was found after exposure to 10% CSE (B), while not DNA damage was found for CSE (C). The results show induction of oxidative DNA damage by filterless CSE and a protective effect of filter for cigarettes B and C, pointing out the suitability of this experimental model to sensitively detect oxidative effects of complex inhalable mixtures on target organ.

## EFFECTS OF NANOSIZED CARBON BLACK (NCB) ON MOLLUSCAN IMMUNOCYTES

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Nanotechnology has become one of the leading technologies over the past 10 years for the unique physical and chemical characteristics of nanosized materials (Oberdöster G. et al., 2005). With increasing industrial production of nanomaterials, public concern on their environmental and health effects is growing rapidly. Industrial products and wastes tend to end up in waterways therefore it is inevitable that nanoscale products will enter the aquatic environment: in this light, the effects of nanoparticles on aquatic organisms can represent a major concern. One of the most widely studied and used nanomaterials is carbon black (NCB), that has widespread applications in both industrial and domestic products. In this work, the effects and possible mechanisms of toxicity of NCB were investigated by multiparametric in vitro assays (Betti M. et al., 2005) in the cells of an aquatic invertebrate, the marine mussel *Mytilus galloprovincialis.* Mussel immune cells (hemocytes) were utilised to evaluate the effects of short-term (0–60 min) and low dose (1–10 μg/ml) exposure to NCB. NCB does not induce significant lysosomal membrane destabilization; on the other hand, NCB significantly stimulates lysosomal enzyme release, phagocytic activity, NO release and extracellular and intracellular superoxide ($O_2^-$) production, indicating inflammatory processes (Delaporte M. et al., 2006). Moreover, flow cytometry shows that NCB treatment seems to compromise mitochondrial function, being evident as

loss of mitochondrial membrane potential and a decrease in mitochondrial mass/number. The results demonstrate that molluscan immunocytes represent a suitable model for evaluating the effects of nanoparticles in aquatic organisms.

## EVOLUTION OF X-RAY INDUCED DNA DAMAGE IN MOUSE SPERMATOGENESIS

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DNA integrity in sperm is essential for the accurate transmission of the paternal genetic information and the consequent maintenance of healthy future generations. It has recently become evident that, also in the semen from fertile individuals, a fraction of spermatozoa carrying defective DNA is always detectable. It is speculated that DNA damage can be the result of testicular apoptotic-like events or can be produced during spermiogenesis whenthe DNA must be packedaging around the new protamine core. In order to evaluate whether mature spermatozoa DNA changes alterations could stem from DNA damage induced in immature germ cells, testis cells and spermatozoa were analysed by the neutral comet assay immediately, 2 hours, 21, 33, 45 and 100 days after in vivo irradiation with 4 Gy X-ray.

As expected, X-rays induced DNA double strand breaks (dsb) in testicular cells as evidenced by the increase of comet parameters immediately after treatment. These lesions were no more detectable within 2 hours after irradiation. On the contrary, a significant increase of comet parameters was again observed after 33 days. This increase was especially evident in the elongated spermatid subpopulation. DNA damage was still present although decreased at 45 days although at a lower level. At this time, a consistent increase of damage parameters was also observed in spermatozoa. 100 days after irradiation (more than 2 spermatognic cycles) the comet parameters returned to control values both in testis and sperm cells.

These overall data suggest that testis cells repair double strand breaks dsb as rapidly as somatic cells but, at the same time, when damage is induced in a limited premeiotic stage, a comet undetectable chromatin alteration persists during the successive mitotic and meiotic phases eventually and it is converted into strand breaks during spermiogenesis and thus becoming detectable in the latest stages of spermatogenesis.

## HL60 HUMAN PROMYELOCITIC CELL LINE, A MODEL SYSTEM FOR OXIDATIVE DAMAGE STUDIES

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HL60 cell line, isolated from the blood of a patient affected by promyelocitic leukaemia, has 45–46 chromosomes with abnormalities mainly on chromosomes 5, 8 and X and can undergo in vitro differentiation. Several chemicals can induce such processes: di-methyl sulphoxide (DMSO) and retinoic acid can induce granulocytic differentiation while vitamin D3 and phorbol esters (PMA) treatments can give rise to the formation of monocytes and macrophages. We have characterized these cells before and after differentiation induction analyzing endpoints particularly useful in the study of oxidative damage. In our experiments we define as proliferative cells (AP) HL60 in log phase of growth with less than 10 passages from defrosting, as differentiated (D) cells treated with 10 nM PMA for 72 hours. We have evaluated: the expression of differentiation cluster membrane antigens (CD95, CD9, CD1A and CD14), the order and the structure of membrane, the nuclear structure, the level of intracellular reactive oxygen and nitrogen species (ROS and RNS) and the intracellular concentration of glutathione (GSH). The methods employed are all based on the use of fluorescent probes and techniques, such as the Comet Assay for the evaluation of DNA integrity, the Generalized Polarization for the membrane order and the citofluorimetric analysis for the cell cycle and the ROS and RNS evaluation. The GSH concentration has been measured by a colorimetric-enzymatic assay. The results show that: (i) after differentiation induction the cells loose their ability to proliferate (more than 80% of the cells are in G0 phase), (ii) there is an increase in the D cell membrane order and rigidity compared to AP cells, (ii) the nucleus of D cells is more compact and less prone to oxidative damage than that of AP cells (iii) D cells show a lower intracellular concentration of ROS and RNS compared to AP cells while (iv) the opposite is true for the intracellular concentration of GSH and finally (v) AP cells are positive for CD95 and negative for CD1A, CD9 and CD14 while D cells are positive for CD9 and CD1A and negative for CD95 and CD14. These experimental evidence show that HL60 can be a valuable cellular system to study the cell reaction to oxidative stress as a function of metabolic and structural changes linked to differentiation processes, acting within few hours, and aging phenomena mainly due to the accumulation of damages on long time scale.

## DIFFERENTIAL RESPONSE TO OXIDATIVE DNA DAMAGE IN SPERM AND SOMATIC CELLS

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Damage that engenders DNA double strand breaks activates Ataxia Telangiectasia Mutated (ATM) kinase through its auto- or trans-phosphorylation on Ser1981. Activated ATM (αATM) is one of the mediators of histone H2AX phosphorylation on Ser139. The phosphorylated histone, known as γH2AX, is assumed to change local chromatin structure, enabling the recruitment of other DNA repair proteins. Several studies indicated that γH2AX could be used for evaluating DNA damage produced by a variety of chemicals/stress factors in somatic cells. A recent work by Li et al. (2006) reported that γH2AX evaluation by flow cytometry (FCM) can be a useful tool for scanning sperm DNA damage induced by oxidative stress. The aim of our

study was to investigate the recruitment and phosphorylation of ATM and H2AX in human sperm and in the human lymphoblastoid cell line RPMI 1788 undergoing $H_2O_2$ treatment.

Sperm and somatic cells were treated with different concentrations of $H_2O_2$ (0.0, 1.0, and 10.0 mM). Afterwards, αATM and γH2AX were checked by FCM fluoroimmunodetection. We also used both alkaline and neutral comet assay for assessing the occurrence of single (SSBs) and double DNA strand breaks (DSBs), respectively. Our results evidenced that neither αATM nor γH2AX were evoked in sperm cells after $H_2O_2$ treatment. Neutral comet assay could not reveal DSBs, whereas the alkaline version detected a dose dependent DNA damage induction. Conversey, both αATM and γH2AX were observed in somatic cells together with the induction of both DNA SSBs and DSBs. These results demonstrate that oxidative stress can induce different effects and responses in somatic and sperm cells, the latter characterized by remarkable chromatin remodeling and massive DNA packaging which deprives the latest stages of mammalian spermatogenesis of effective DNA repair systems.

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DEFINITION OF REES $DL_{100}$ ON *AGROBACTERIUM* SPP. AND *RHIZOBIUM LEGUMINOSARUM FRANK* CULTURES

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Rare Earth Elements (REEs) are naturally present in the environment and in biological systems, but little is still known about the effect of their agricultural application on the growth of soil microorganisms. We investigated by flow cytometry the growth of some bacteria strains, very important in agricultural science, in presence of increasing levels of either a mix of REEs nitrates or lanthanum nitrate alone by an in vitro assay miniaturized procedure. We used it to fix the dangerous range of REEs nitrates and Lanthanum nitrate concentrations and their $DL_{100}$ on bacterial viability, evaluated by flow cytometry, and then we compared the results with those obtained by in vitro standard culture (agar medium in Petri plate). We also tried to study, by Biolog technology, the changes in metabolism which occurred when bacteria are treated with a critical concentration of REEs nitrates and lanthanum nitrate alone.

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BORON NEUTRON CAPTURE THERAPY (BNCT): CHARACTERIZATION OF THE RESISTANT CELL FRACTION

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The boron neutron capture therapy (BNCT) is a targeted radiotherapeutic approach for cancer treatment based on the cytotoxic effects of highly ionizing particles yielded by the reaction of neutrons with $^{10}B$ atoms. Its efficiency is tightly related to the levels of $^{10}B$ selectively uptaked by tumor cells *vs* normal ones. At the present time, boronophenylalanine (BPA) and sodium borocaptate (BSH) are the only two boron delivery agents clinically used. Our clinical applications of BNCT, on patients affected by diffused liver metastases, were performed using BPA as boron carrier. Despite the positive oncological efficacy of this therapeutic approach, the appearance of recurrencies of the tumour in the long survivimg patient some years after the treatment gives evidence in favour of the existence of an even though minimal cell fraction refractory to BNCT. We suppose they are quiescent cells, unable to capture sufficient levels of the aminoacidic compound BPA, at the time of treatment. With the aim to support such hypothesis, exponentially growing DHDK12TRb coloncarcinoma cells were cultured for four hours in medium supplemented with BPA, at concentrations ranging from 10 to 160 ppm, in addition with 10 mm BrdU. After harvesting and counting, cells were exposed to neutron irradiation and reseeded, at all the programmed concentrations, in the bottom of glass Petri dishes for plating efficiency test and for BrdU immunostaining. Fluorescence microscopy of surviving clones failed to show any BrdU labelling thus confirming the suggested hypothesis that these cells belong to the non proliferating compartment of the original in vitro culture.

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QUALITY ASSESSMENT OF REFRIGERATED EQUI-NE SPERM USING INNOVATIVE METHODS

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The effect on equine sperm of overnight refrigeration, a preservation method widely used in reproductive clinics, was estimated by innovative methodologies. Eleven semen samples fresh and diluted with INRA 96 extender were analysed for viability (propidium iodide), for chromatin structure stability (SCSA) and mitochondrial membrane potential (JC-1) using flow cytometry, for motility using CASA. After refrigeration, sperm viability dropped from 99% to 33%. A decrease in motility parameters was observed, particularly in progressive motility (40% vs 5%). SCSA analysis showed a 2–3 fold increase in DNA Fragmentation Index percentage (DFI) over fresh samples. Refrigeration caused a significant decrease of mitochondrial membrane potential measured as reduction in the numbers of sperms with orange fluorescence (JC-1 aggregates). Preliminary evaluations of sperm mitochondrial distribution by confocal microscopy with Mito-Tracker Orange were carried out on two fresh samples and active mitochondria were visualized in intermediate tract. This use of non-subjective methodologies enables a decrease in refrigerated sample quality to be highlighted.

## CYTOGENETIC BIOMONITORING OF HOSPITAL PERSONNEL HANDLING ANTINEOPLASTIC DRUGS

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Antineoplastic drugs constitute a vast and heterogeneous group of compounds of different origins with different chemical structures known for their potentially mutagenic, carcinogenic and teratogenic effects.

Occupational risks to persons handling these mutagenic/carcinogenic substances, such as workers in drug manufacturing, pharmacists in drug preparation, nurses administering drugs and physicians and nurses in patient care, has led to health concern. Although health workers are exposed to much lower doses compared to cancer patients, low-dose exposure over a long period of time can have long-term effects on the worker's health.

Safety guidelines and recommendations have been published in several countries in order to improve operating procedures and keep exposure levels as low as possible. Nevertheless, contamination in the working environment can still occur.

The aim of the present multicentric study was to monitor the genotoxic risks associated to antineoplastic drugs handling in nurses from 5 Italian hospitals by evaluating the frequencies of micronuclei and chromosome aberrations in peripheral blood lymphocytes.

The preliminary results reported in this work are referred to 30 exposed subjects and 30 controls. Our data showed that occupational exposure to antineoplastic drugs is associated with an increased frequency of both micronuclei and chromosome aberrations.

## CYTOMIC MINIATURIZED ASSAYS OF OXIDIZED DNA BASE 8-OXOGUANINE AS AN INDICATOR OF OXIDATIVE STRESS

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Oxidative damage to DNA is related to mutagenesis, carcinogenesis and cytotoxicity by xenobiotics. 8-oxoguanine (8-oxoG) is a major product of oxidative DNA damage and a useful marker of DNA oxidation. Detection of 8-oxoG by chromatography is complex and time-consuming. High-content bioimage assays (HCA) and flow cytometry (FCM) allow single-cell multiparametric fluorescence measurements of heterogeneous cell populations for analysis of cytotoxicity in vitro. The purpose of this work was to provide fast and simple complementary HCA and FCM assays for studies of oxidative damage to DNA. The assays were performed on hepatic (HepG2), neuronal (SH-SY5Y) and renal (A704) cells growing on 96-well plates and acutely exposed to methylene blue plus light (positive control) and to 25 reference chemicals. We used the OxyDNA Assay (Calbiochem) for detection of oxidative damage to DNA, based upon the binding of a FITC-labelled antibody to 8-oxoG epitopes in DNA of fixed cells. For HCA, cells were fixed in situ, stained with anti-8-oxoG antibody and nuclei counterstained with DAPI. HCA was performed with an InCell Analyzer 1000 (GE Healthcare) using appropriate algorithms for cell segmentation and data calculation. For FCM, cells remained attached during toxicant exposure and were trypsinized and resuspended for fixation, fluorescent staining and analysis in a 96-well plate-loading flow cytometer (Cytomics FC500 MPL, Beckman-Coulter). Both assays detected dose-dependent DNA damage induced by positive controls and allowed quantification (EC50) of cytotoxic effects of different compounds inducing DNA oxidative damage. FCM and HCA provide complementary information and allow a consistent detection and quantification of oxidative effects on DNA.

## FISHING CEREALS CHROMOSOMES: USING FLOW CYTOMETRY AND SORTING TO REVEAL GENOME ORGANIZATION IN WHEAT

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Cereals are the most important crop for nutrition and account for more than 1000 million metric tons in 2006. Durum and bread wheat world production has been of about 590 million tons in 2006 ranking wheat as the top producer among cereals. Biotic and abiotic stresses, such as pests and drought or salinity, respectively, they have both a strong impact onto this species and its genetic improvement is of crucial relevance for global food and feed production. An international consortium has been developed to coordinate whole wheat genome sequencing, the largest genome sequencing initiave never ever attempted. Both durum and bread wheat hold a very large genome (12Gbp and 17Gbp about, respectively) made of more than 80% of repetitive DNA sequences of polyploid origin. Its genetic analysis is hampered by the large size and the presence of homeologous genomes (A + B and A + B and D, for pasta and bread wheat, respectively). One of the ways to simplify genome analysis in wheat is to create specific resources for the three genomes by a single chromosome approach combined with flow sorting.

However, due to the lack of sufficient differences in size between individual chromosomes in durum and bread wheat, only chromosome 3B could be sorted into a high-purity fraction from standard lines. Genetic stocks such as aneuploids are an extremely usefull material to allow chromosome flow karyotyping and sorting. Ditelosomic lines have been used for flow sorting of a single arm and construction of first chromosome specific BAC libraries. We have tryied to take over such a limitation in chromosme discrimination developping a tool which allow for chromosome characterization (banding) and isolation on the basis of FISH labelling of chromosomes In Suspension (ISFISH) and fluorescence-activated sorting of chromosome fractions. Banding in suspension was achieved after alkali chromosome DNA denaturation and hybridization with a biotin labelled GAA microsatellite probe. Our results demonstrate the potentialities of ISFISH for chromosome flow karyotyping and sorting of single type chromosome suspensions from standard lines, using suitable probe.

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CYTOMETRY AND INNOVATION, THE imPACT PROJECT

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Conventional flow cytometers have intrinsic electronic limits represented by the way the signals are processed and stored. The signals are processed by "peak and hold" and the A/D converter digitizes only the held peaks; the pulse shape is not stored and all informations about the original one is lost. More, the log scale is affected by "offset and scaling factor" related to the instrument model. Finally, the instrument setup and data analysis are very dependent on the operator arbitrary settings. The **i**ntelligent **m**onitoring **P**ortable **A**ctive **C**ytometry **T**erminal (**imPACT**) is a flow cytometer prototype which couples portability and hi-grade automation by electronic and optical innovation, overcoming these drawbacks. The prototype is designed to be extremely portable, to operate in any condition, to work in full automatic mode and to. transmit data strings by satellite or ground TLC network to a remote workstation. The Hw/Sw hybrid (SoftSTONE) interacts with front-end programmable electronics, so to be integrated in a distributed network of marine cytometry active terminals.

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IN VITRO CYTOMIC ASSAYS OF EMBRYOTOXICITY USING MURINE EMBRYONIC STEM CELLS

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New in vitro methods alternative to animals for prediction of human toxicity are demanded by the European Comission to assess the chemical risk of thousands of industrial compounds (REACH policy). Current regulatory embryotoxicity tests in vitro or in vivo are time-consuming and require complex cellular systems or sacrificing pregnant animals. The use of embryonic stem cell lines reduces the use of animals and allow testing toxicity during differentiation. We have applied flow cytometry to the murine embryonic stem cell line D3 (D3-ES). These cells maintain their pluripotent undifferentiated status in presence of leukaemia inhibitory factor (LIF). D3-ES cells are cultured in 96-well plates in presence or absence of LIF plus the test chemical. Cytotoxicity is tested by viability test with propidium iodide. Toxics were selected on the basis of their already predicted human acute toxicity or their lack of correlation between in vivo and in vitro basal cytotoxicity assays. IC50 was determined for each compound. Our results revealed differences in the effect of toxics on D3-ES cells cultured with or without LIF, i.e., in indifferentiated or early differentiated stages. Our study may be the basis of a technological platform for the prediction of potential chemical embryotoxicity, combining cytomic technology with murine embryonic stem cell lines.

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GENETICAL DETERMINANTS OF DETOXIFICATION - SUSCEPTIBILITY TO SEVERAL DISEASES AND CANCER

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Individual susceptibility to cancer results from several exogenous and endogenous factors including differences in metabolism and nutritional status. Most carcinogenes must undergo metabolic activation to form electrophilic reactans which can bind to DNA. Consequently individual features of carcinogen metabolism play a primary role in susceptibility to environmental diseases and cancer. The genetic polymorphism have been detected in a variety of phase 1 (activating), mainly cytochrome P450-related enzymes (CYPs), and phase 2 (inactivating) enzymes as glutathione-S-transferases (GSTs) and N-acetyltransferases (NATs), involved in detoxification of chemical carcinogens.

In our laboratory for preventive DNA diagnostics we perform tests with isolated DNA from dried saliva samples, using PCR and RFLP technics.

Genetic variants of a number of polymorphic metabolic enzymes are analysed: e.g. CYP1A1, CYP1A2, CYP2E1, several GST genes, NAT genes, SULT1A1 and mEH. The assessment of a single polymorphic genotype cannot be expected to be sufficient for evaluating individual risk, because of the combined effect of metabolic genes in disease proneness: therefore, a detailed profile for each patient is preferable. The determination of metabolic at-risk genotypes should allow persuade at-risk patients to change their way of life eliminating their potential habits correlated to cancer occurrence, for example smoking and unbalanced diet.

## ASSESSMENT OF LYCOPENE'S ANTIOXIDANT ACTIVITY ON AN MCF-7 CELL LINE USING COMET ASSAY AND ROS EVALUATION

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Lycopene (Lyc), a naturally-occurring molecule in tomatoes, is known to protect against oxidative stress. No data are available on the dose-response relationships between lyc and antioxidant activity and between ROS determination and DNA damage. We studied lyc's antioxidant activity on basal reactive oxygen species (ROSb) using MCF-7 cells and a DCF-DA probe (5 μM). Fluorescence was measured using flow cytometry (FC) and a plate reader. A slight reduction (up to 18%) in ROSb was recorded by plate reader in MCF-7 cultures treated with 20 μM lyc while using FC a greater reduction (∼37%) in ROSb was observed on cells treated with 0.2 nM lyc. The COMET assay showed no modifications in tail length compared with the negative control ($-H_2O_2$) following exposure to lyc. After oxidative stress with $H_2O_2$ (10 μM) both fluorescence recorders detected a reduction (∼23%) in ROS (ROSi) compared with the positive control ($+H_2O_2$). No DNA protection was observed in MCF-7 cells exposed to oxidative challenge and to lyc. The different effect of lyc on ROS and DNA could be explained by lyc's mild antioxidant activity on both ROSb and ROSi.

## SCREENING OF FLUORESCENT PARTICLES IN TURBID MEDIA AN INNOVATIVE TECHNIQUE

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In a collaboration between the University of Illinois at Urbana-Champaign and the Pluristandard company a technique was developed that allows rapid screening of fluorescent particles in turbid media. The particles could be fluorescently labeled cells, bacteria, viruses or protein aggregates and large macromolecules. The method is fast and reproducible and it could be utilized for the diagnosis of the initial phase of an infection or for the evaluation of pathogens in food and in water. The sensitivity is very high, only limited by the amount of the sample that can be analyzed. This amount depends on the total measurement time. In our preliminary measurements we were able to count the particles present in a volume of about 1 ml in 100s.

The purpose of these preliminary measurements was to establish the sensitivity and the reproducibility of the method. The samples were prepared by adding either the cells or the bacteria to a buffer solution with added scattering particles to reproduce realistic experimental conditions.

For the experiments described in this poster, we used real biological samples with a preparation procedure that could be used for routine measurements. In particular we examined two types of samples. In one, we measured the number of somatic cells in cow milk; in the second we measure the total concentration of bacteria in urine samples.

In both cases we determine the dilution ratio necessary to bring the particle counts into the linear region of the instrument response where we have the maximum sensitivity. We discuss our results and we compare our determinations with results of traditional methods.

## LIGHT-DEPENDENT PRODUCTION AND GROWTH RATES OF FRESHWATER PICOCYANOBACTERIA ARE STRAIN-SPECIFIC AND SENSITIVE TO PHOTO-ACCLIMATION

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We investigated the effect of different light conditions on primary production and growth rates of 3 closely related freshwater picocyanobacterial strains from 3 ribotypes in cultures. We compared two phycocyanin strains (PC) and one phycoerytrin strain (PE) isolated from subalpine lakes. Experiments were conducted over 9 light intensities (LI) ranging 6–1500 μmol $m^{-2}$ $s^{-1}$ with cultures that were acclimated to low and moderate irradiance. Primary production (3 replicates, 4h incubation) was measured by uptake of $^{14}C$, and growth rates were estimated by flow cytometry (BD FacsCalibur). The results revealed strainand pigment-type-specific significant differences in growth and production rates. The PE was sensitive to high light conditions and reached highest photosynthesis and growth rates at low LI. In contrast to the PC, the photoacclimation of PE to moderate LI reduced its photosynthesis and growth rates. Growth rates differed widely in response to irradiance and photoacclimation, ranging from negative values measured at the lowest and highest irradiance to positive rates of up to 0.9 $d^{-1}$ measured at moderately high light levels. Photoacclimation significantly affected both primary production and growth rates of all three strains investigated.

## HPLC-UV ANALYSIS OF ARTEMISININ CONTENT IN DIFFERENT CULTIVAR OF *ARTEMISIA ANNUA* L. DURING THE VEGETATIVE CYCLE

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Artemisinin is an antimalarial compound present in *Artemisia annua* L. and is very effective against drug resistant Plasmodium species. Artemisinin is an oxidized sesquiterpene lactone with an "endoperoxide bridge", that seems to be responsible of its antimalarial activity, and has mainly been detected in the aerial parts of the plant, in particular in the leaves and flowers.

As Artemisia has been proposed as an alternative crop in the areas of Campania liable to the conversion of tobacco, a preliminary study about the accumulation of artemisinin in the plant, during all vegetative stages, was performed, comparing three cultivars grown and two planting density. The trial was carried out in the experimental field of "CRA-Istituto Sperimentale per il Tabacco" in Scafati (SA), using a split plot design with two replicate blocks. During the life

cycle of the plant until flowering, every two weeks, the aerial parts (leaves and stalks) were harvested separately, weighed, dried in an air-forced oven at 60°C, grounded and analyzed by HPLC with UV detection method.

Artemisinin is UV-transparent and a derivatization procedure for HPLC-UV analysis was required, which involved the conversion of artemisinin in a strongly UV-absorbing compound, named Q260. The procedure is based on the artemisinin hydrolysis with sodium hydroxide, giving a compound called Q292, which was further converted into Q260 by acidification.

Preliminary data on the growth of the plant revealed that the planting density had a light positive effect on the development in height; the highest increase in plant dry weight has been observed between 90 and 105 days from the transplant. Moreover it was observed that the dry weight percentage of the leaves on the plant, for all varieties, decreased with the growth of the plant.

Artemisinin content in leaves increased during the vegetative cycle and revealed a different behaviour among cultivars, in particular Crono and Pericles reached the highest artemisinin values after 105 days from transplanting, while Eureka cultivar after 75 days. As regards the effect of planting density on artemisinin content the highest values were always observed at the lower density. Artemisinin content in the stalks was negligible.

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COMPARISON BETWEEN FLOW CYTOMETRIC AND MICROSCOPIC ANALYSIS OF H2AX HISTONE PHOSPHORYLATION IN BONE MARROW AND TESTICULAR CELLS OF MICE AFTER X-RAY IRRADIATION

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H2AX histone phosphorylation is a marker of radiation-induced DNA damage. We compared different immunocytochemistry methods to measure H2AX phosphorylation (γH2AX) in bone marrow and testis cells of mice irradiated with 1–4 Gy X-rays and killed 0.5–48 h after irradiation. By microscopy, the percentage of γH2AX+ bone marrow cells peaked at 1 h and returned to control 24 h after irradiation. By flow cytometry, a kinetics of the percentage of γH2AX+ cells was described parallel to microscopic data, whereas the time course of the mean γH2AX fluorescence intensity per cell showed a slower return to control values, suggesting longer persistence/later appearance of massive H2AX phosphorylation in few cells. In testis samples scored by microscopy, spermatocytes (SC), round (RS) and elongated spermatids (ES), recognized by nuclear morphology, were separately analyzed. Less than 1% of ES were labelled, irrespectively of treatment. γH2AX staining of the sex vesicle in pachytene SC was specifically detected and did not hinder the analysis of radiation effects. Irradiation significantly increased the percentages of γH2AX+ RS and SC that, unlike in bone marrow, persisted from 1 to 48 h after irradiation. By flow cytometry, higher frequencies of 1c- and 4c-labelled cells were detected in unirradiated testis than by microscopy, because of sex vesicle labelling and possibly because untreated RS contained phosphorylated histone molecules not aggregated in a microscopically detectable pattern. A radiation effect was also detected by flow cytometry 1 h after irradiation, but, unlike for microscopy, the parameters were back to control at 24 h, suggesting that in germ cells irradiation induced, more than phosphorylation of H2AX, a redistribution of pre-existing phosphorylated molecules only detectable by microscopic analysis.

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COMPARISON OF THREE METHODS TO ESTIMATE PHYTOPLANKTON LYSIS RATES

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There is growing evidence that cell death and subsequent lysis of phytoplankton are common and relevant phenomena at sea, resulting from a number of factors, including parasitic attack by viruses, or exposure to physiological extremes of light, temperature and nutrient concentrations. However, the importance of phytoplankton lysis to phytoplankton losses in the sea is still poorly understood, mainly due to the lack of reliable techniques to quantify this process.

We compared three methods that are commonly used to estimate phytoplankton lysis: (1) fluorescein diacetate cleavage by intracellular esterases which are released by dead cells; (2) vital staining by SYTOX Green, which penetrates only cells with disrupted membranes and whose fluorescence was detected by flow cytometry; (3) enzymatic cell digestion by trypsin and DNAse I which only act on cells with damaged plasma membrane, whose concentrations were determined indirectly by flow cytometry. Lysis rates were estimated during the growth curve of a marine diatom culture of *Skeletonema marinoi.* Lysis rates obtained using the three methods followed the same trend, they remained very low in exponential phase and increased exponentially from the stationary to the declining phase of growth. Values obtained from the enzymatic digestion of dead cells and those from the esterase method were very similar, while those obtained from the nucleic staining were twice lower. These results indicate that different processes are involved during membrane disruption occurring during cell death in phytoplankton, esterase release being upstream with respect to membrane permeabilization.

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INFLUENCE OF GLUTATHIONE-S-TRANSFERASE ON DNA DAMAGE INDUCED BY TOBACCO SMOKE

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Genotoxicity of tobacco smoke has long been investigated and tobacco smoke is considered to be one of the principal human carcinogens. Many chemical constituents of tobacco smoke are enzymatically metabolized by phase-I and phase-II enzymes, and modifications in coding and regulating sequences of these genes could influence their ability to

detoxify these compounds. In this work, we studied several enzymes involved in the metabolism of xenobiotics, viz. the glutathione *S*-transferases (GST)M1, T1, P1 and A1, with respect to their influence on the genotoxic effects induced by cigarette smoking. We assessed the genotoxic effects of tobacco smoke on peripheral blood lymphocytes of 72 healthy caucasians by use of the chromosomal aberration (CA) assay and the micronucleus (MN) test. Genotypes of GST M1, T1, P1 and A1 were determined by means of the polymerase chain reaction and methods based of restriction fragment length polymorphism (RFLP). We found that smoke and gender are the two variables that most influence the DNA damage. In particular, we observed that female smokers seem to be more sensitive than male smokers, having a significantly higher frequency of CAs. Moreover, a significant increase in frequency of micronuclei in bi-nucleated cells (BNMN) was found in smokers, but not in non-smokers. This increase seems to be influenced not only by age and gender, but also by genetic constitution. Subjects carrying *GSTM1-null* genotype seemed to have an higher susceptibility to DNA damage induced by tobacco smoke than *GSTM1*-positive ones. When considering a combination of GST genotypes, we found a lower BNMN frequency in subjects with *GSTP1* variant allele plus *GSTM1*-positive genotypes, while the most damaged cells are found in subjects bearing *GSTM1-null* plus *GSTP1*-wild type. Our results suggest that investigation of the association between several gene polymorphisms and important endpoints of DNA damage could contribute to better understanding the role of gene-gene interaction.

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ASSESSMENT OF REPRODUCTIVE CYCLE IN COMMON SOLE BY CITOMETRY

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The common sole Solea solea (L., 1758) is one of the most valuable resources and it is a target fishery species in Mediterranean water, but guidelines about the resource management are generally lacking. In order to increase the scarce knowledge on the annual maturative cycle of the ovary, in the Adriatic Sea, histological and cytological analyses of ovary obtained from monthly captures of sole were carried out. Cytometric analysis was carried out by a computerized image analysis system Leica Qween. The gonad-somatic index (GSI determined on the basis of the ovary to body weight ratio), supported by histological and cytological data, describe the spawning period from December to March. The GSI increased rapidly during the reproductive season, when the majority of oocyte growth (diameter > 200 mm) occurred. The range of germinal cells is comprised between 8 mm (oogonia) and 1.360 mm (hydrated oocytes immediately before spawning). The cytological study of oocyte size distribution showed only previtellogenic oocytes (diameter < 200 mm), during the period April-November, but also vitellogenic oocytes (diameter > 200 mm), during the period December-March. Before ten years ago, the oocyte size distribution in the mature ovaries was rarely studied in populations of common sole and the data obtained were often conflicting. In conclusion, the cytological approach applied throughout the ovary maturation cycle in common sole is a good "assessment environmental test" as gonad maturation is closely dependent on environmental conditions.

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FLOW CYTOMETRY ASSESSMENT OF THE PHYTOTOXIC ACTIVITY OF A NEW TOXIN PRODUCED BY PHYLLOSTICTA CIRSII, A BIOCONTROL AGENT OF CIRSIUM ARVENSE

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A flow cytometric method has been applied for the evaluation of the phytotoxic activity of a new toxin purified from the culture filtrates of Phyllosticta cirsii, a fungal pathogen of Cirsium arvense, one of the most invasive weeds in temperate areas. Our technique has bene developped using Nicotiana tabacum leaf protoplasts as a model organism in comparison to classic microbiological bioessay. Protoplasts were treated with the pure phytotoxin at different concentrations and times of exposure. and then stained by fluorescine diacetate to be analyzed by flow cytometry. The pure metabolite proved to have a fast toxic effect on tobacco protoplasts, which was dose-dependant. The flow cytometry method showed a high sensitivity to small toxin amounts and could also give useful and fast information on the mechanism of action of phytotoxins.

## HEMATOLOGY

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SIX COLOUR FLOW CYTOMETRY AS A CLINICAL TOOL FOR NON-HODGKIN LYMPHOMA DIAGNOSIS

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Diagnosis of non-Hodgkin's lymphoma (NHL) is classically based on histologic analysis, flow cytometry playing an ancillary role. In this study we evaluated the possibility of contributing to NHL diagnosis by using 6-colour flow cytometry (BD FACSCanto II) analysis in clinical practice. In the last 7 months, we investigated 337 specimens from patients with diagnostic suspicion of NHL, comprising bone marrow aspirates (N = 150), peripheral blood samples (N = 134), fine needle aspiration cytology (FNAC) specimens (N = 31), lymph node or gastrointestinal biopsies (N = 10), body fluids (N = 12). We used the following panel, according to the scheme FITC - PE - PerCP (or PerCP-Cy5.5) -

PE-Cy7 - APC - APC-Cy7. First tube: CD43, CD10, CD19, CD20, CD5, CD45; 2nd tube: CD38, CD23, CD19, CD56, CD11c, CD45; 3rd tube: CD103, CD22, CD19, CD25, CD14, CD45; 4th tube: κ-chain, λ-chain, CD19, CD20, CD5, CD45; 5th tube CD4, CD8, CD45, CD5, CD2, CD3; 6th tube: CD1a, CD7, CD45, CD56, CD2, CD3; 7th tube: TCRαβ, TCRγδ, CD45, CD5, CD2, CD3. Cytometric diagnosis was compared with histologic results and final diagnosis. The contribution of flow cytometry was critical in 100% of bone marrow and peripheral blood samples. Flow cytometry evaluation of FNAC specimens adequately supported the diagnosis of FL in 11 cases (36%), MZL in 6 cases (19%), MCL in 2 cases (6%), SLL in 1 case (3%) and T-cell NHL in 1 case (3%). Light chain restriction (κ or λ) was demonstrated in 17 cases of 20 B-NHL (85%). Ten FNAC specimens were considered as reactive hyperplasia (33%). These cases included Hodgkin's lymphoma patients. Flow cytometry evaluation of biopsy specimen supported the diagnosis of MZL in 2 cases and T-cell LNH in 2 cases; 6 biopsy specimens were considered as reactive hyperplasia. This study demonstrates that 6-colour flow cytometry is suitably applicable, on a routine basis, to NHL diagnosis.

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PLATELET-LEUKOCYTE CONJUGATES IN PERSISTENT ATRIAL FIBRILLATION (AF)

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AF is the most common sustained arrhythmia in clinical practice; platelet abnormalities, as well as inflammation have been associated with AF and may increase the risk of thromboembolism. Activated platelets may recruit polymorphonuclear leukocytes (PMNs) and mononuclear leukocytes, contributing to the pathogenesis of thrombotic complications.

Eleven patients with persistent AF, not on anticoagulant therapy, were studied in comparison to 28 controls in sinus rhythm, frequency matched for age and sex. Platelet-leukocyte conjugates, platelet P-selectin (CD62P) and leukocyte CD11b expression were measured by flow cytometry in citrated whole blood in basal conditions and upon stimulation with ADP (2 μM)/collagen (2 μg/ml) and reported as percent of positive cells. Medians were compared by Mann-Whitney U test for intergroup differences.

In AF patients, platelet-PMN conjugates did not differ from controls in basal conditions (2.4 *vs* 2.8 %, median values), while the expression of CD62P was significantly lower (1.3 *vs* 3.6 %, median values, $p = 0.018$). After ADP/collagen in vitro stimulation, platelet-PMN conjugates were significantly lower in AF patients *vs* controls (4.1 vs. 15.3 %; $p = 0.006$) and CD62P expression showed a trend to decrease (16.9 *vs* 23.6 %). PMN CD11b was not overexpressed in AF, either in basal conditions or upon stimulation, an observation in agreement with a virtually unmodified intracellular myeloperoxidase content.

In conclusion, platelets were less reactive in AF in comparison to controls, as shown by reduced CD62P expression and interaction with PMN, while leukocyte function did not seem to be significantly modified.

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ADENOSINE INHIBITION OF ADP-INDUCED MONOCYTE-PLATELET AGGREGATES FORMATION IN CARDIAC SYNDROME X

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Previous studies showed an increased formation of monocyte-platelet (mono-plt) aggregates in response to adenosine diphosphate (ADP) at rest in patients (pts) with cardiac syndrome X (CSX) compared to healthy subjects. ADP response was reduced after exercise, possibly indicating an involvement of adenosine, typically increased following exercise. To directly test this, in the present study, we assessed whether adenosine may prevent ADP-induced mono-plt aggregate formation. We studied 7 CSX pts (M/F 4/3; age 50 ± 5) and 7 healthy subjects (M/F 4/3; age 54 ± 3). Mono-plt aggregates were assessed by flow cytometry at baseline and after ADP stimulation ($10^{-7}$M) with and without pre-incubation with physiological amounts of adenosine ($10^{-5}$M). Mono-plt aggregates were identified using the logical gating facility by combination of binding characteristics of anti-CD14 and of anti-CD41. At baseline, there were no differences in the percentage of mono-plt aggregates and CD41 expression (an index of monocyte-bound plt number) between the two groups ($p = 0.3$ and $p = 1.0$, respectively). Following ADP stimulation, mono-plt CD41 expression significantly increased in CSX pts ($p = 0.04$) and in controls ($p = 0.02$). In contrast, ADP increased the percentage of mono-plt aggregates in CSX pts ($p = 0.03$) but not in controls ($p = 1.0$). All the enhancing effects of ADP were abrogated by adenosine. Thus, exercise-induced adenosine may play an effective role in reducing ADP-driven mono-plt formation after exercise seen in CSX pts.

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A COMPARISION BETWEEN ISOTYPE CONTROL AND RATIO METRIC METHOD TO DETECT ZAP70 EXPRESSION IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

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The course of B-CLL is heterogeneous and the evaluation of prognosis up to date remains a difficult issue. ZAP70 has been proposed as new prognostic factor, but the interpretation of the results is still controversial.

By flow cytometry, we compared two techniques for ZAP70 assessment, employing the same mAb anti-ZAP70 Alexa Fluor 488: the isotype control and the ratio metric

method. The results were also correlated to CD38 and to the status of somatic hypermutations (SHM). 64 B-CLL patients and 10 healthy subjects were studied.

By isotype control method (using the cut-off 30%), 19 patients (30%) were ZAP70 − ve and 45 patients (70%) were ZAP70 + ve, with normal subjects being ZAP70 − ve. By ratio metric method (using the cut-off 0.30), 9 patients (14%) were ZAP70 − ve and 55 patients (86%) were ZAP70 + ve, with normal subjects being 100% ZAP70 + ve, i.e. the ratio metric method detected an abnormaly high number of ZAP70 + ve cells both in patients and in normal subjects. Considering the lack of ZAP70 expression in normal subjects, we hypothesized that the 0,30 cut-off was too low to discriminate ZAP70 negativity and we increased the cut-off to 0,40. With the new cut-off the healthy subjects were ZAP70 − ve, whereas 28 patients (44%) were ZAP70 − ve and 36 patients (56%) were ZAP70 + ve.

Using the new cut-off, CD38 and ZAP70 significantly correlated ($p < 0.01$), whereas the correlation ZAP70/SHM was never found statistically significant.

Our data highlight the reliability of ratio metric method for detection of ZAP70 positivity but point out the inadequacy of the 0,30 cut-off proposed in the literature.

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## THE NUMBER OF CIRCULATING ENDOTHELIAL CELLS IS NOT INCREASED IN SYSTEMIC SCLEROSIS AND RELATED DISORDERS: ASSESSMENT OF THE PATHOGENETIC ROLE PLAYED BY CXCR4$^{POS}$ PROGENITOR CELLS COEXPRESSING MONOCYTIC AND ENDOTHELIAL MARKERS

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There are ongoing controversies regarding the role of circulating endothelial mature and progenitor cells (CECs/CEPs) in the pathogenesis of the systemic sclerosis (SSc). Circulating hematopoietic and/or endothelial progenitor cells, possibly implicated in the pathogenesis of SSc, were characterized and enumerated by a multiparametric 4-color flow cytometric protocol, in combination with different progenitor cell replating assays, in peripheral blood specimens obtained from 40 patients with SSc, various autoimmune diseases, and controls. Data were compared with patient's clinical features and serum levels of angiogenic factors.

An increased number of CD34$^{pos}$/CD45$^{pos}$/CD184$^{pos}$ (CXCR4) and CD34$^{pos}$/CD45$^{pos}$/CD117$^{pos}$ (c-kit-R) hematopoietic circulating progenitor cells (HCPCs) coexpressing endothelial and monocytic markers was enhanced in SSc patients. No circulating CD45$^{neg}$ endothelial cells were observed. Clonogenic assays confirmed the differentiation of the HCPCs towards hematopoietic-erythroid lineage rather than the endothelial one. In particular the presence of freshly detected CXCR4$^{pos}$HCPC and of in vitro cultured spindle-shaped endothelial like cells (SELC) with an endo/myelomonocytic profile resulted correlated either to SDF-1 and VEGF serum level or to a more fibrotic clinical features of the disease, thus supporting a possible role of these cells in fibrosis.

In SSc patients, the mobilization of in particular endothelial-like CXCR4$^{pos}$HCPCs suggests an unreported contribution of the hematopoietic progenitor cells in the pathogenesis of the disease. Angiogenic factors could play a role in facilitating their organ homing and their perivascular positioning and retention.

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## IgV$_H$ MUTATIONAL STATUS AND ZAP-70 EXPRESSION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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The mutational status of immunoglobulin heavy chain variable (IgV$_H$) genes is considered the best prognostic factor in patients with chronic lymphocytic leukemia (CLL). Lack of IgV$_H$ mutation is associated with rapid disease progression and shorter survival. The zeta-chain of T-cell receptor-associated protein kinase 70 kDa (ZAP-70) has been reported to be a surrogate marker for IgV$_H$ mutation status and its expression in leukemic cells correlates with unmutated IgV$_H$. We report on 82 patients with CLL (mean age 63 yrs; range 40–83 yrs; 41 male and 41 female) in which IgV$_H$ genes and ZAP-70 expression were simultaneously tested at diagnosis. ZAP-70 was evaluated by means of flow cytometry (Crespo et al, NEJM 2003) and positivity was defined as $\geq$ 20% on B-cells. With respect to IgV$_H$ genes, sequences with less than 98 percent homology to the corresponding germ-line IgV$_H$ sequence were considered mutated.

Twenty-five (30%) patients were found ZAP-70+ and 57 (70%) patients ZAP-70−, while, according to their IgV$_H$ mutational status, 55 (67%) patients were mutated and 27 (33%) unmutated. Regression analysis and Pearson's correlation test showed an inverse correlation (r −1.344; p 0.001) between the percentage of IgV$_H$ homology and ZAP-70 cell number. In the ZAP-70+ group, 9 patients were mutated and 16 unmutated, while in the ZAP-70− group, 38 patients were mutated and 19 unmutated (p 0.019). Finally, patients that did not require therapy were more represented in the mutated group ($p < 0.0001$).

In conclusion, our data also confirm that the presence of an unmutated IgV$_H$ is strongly associated with ZAP-70 expression and disease progression.

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## PERIPHERAL CD34+ STEM CELLS INCREASE AFTER MYOCARDIAL INFARCTION

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Introduction: Peripheral stem cell trafficking has been described in a number of ischemic events. The aim of this study was to evaluate the peripheral stem cell CD34+

increase in patients treated by angioplasty after myocardial infarction (MI). Materials and Methods: in 74 patients (P) with MI peripheral blood samples were collected within 24h after diagnosis ($T_0$). In 62/74 patients a second sample was collected within 5–9 days ($T_1$), and in 21/62 patients a third sample was collected within 3 weeks ($T_2$). 22 blood donors (D) were the control group. The samples were labelled with CD34FITC/CD133PE/CD45PerCP, and analyzed by ISHAGE method. The absolute values were obtained by double platform. The stem cell counts were related to some clinical data: the cardiac blush after angioplasty (a good prognostic factor) and the myocardial function recovery after 6 months (REC). Student's t test was applied to evaluate differences. Results: The main results were: a) Large individual variability of CD34+ cells counts both in P and in D was observed (data not shown). b) The mean value of CD34+ count in P-$T_0$ was lower than in D group ($p < 0.0001$), differences between blush and no-blush P were not found. b) in blush-P (but not in no-blush-P) CD34+ count increased at $T_1$ vs $T_0$ ($p < 0.0001$), the CD34+ count of P-$T_1$ vs. D showed no difference. This trend was confirmed for blush-P-$T_2$ too. c) In overall P the CD34+ $T_1/T_0$ ratio >2 showed predictive positive value to the REC = 73.68%, and weak predictive negative value = 48.78%. Conclusions: despite large individual variability, the mobilization of CD34+ is detectable, and it may be of prognostic value in patient treated after myocardial infarction (MI), confirming that CD34+ stem cells may play a role in tissue repair and revascularization.

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FLOW CYTOMETRY IMMUNOPHENOTYPE IS A POWERFUL APPROACH IN MONITORING MINIMAL RESIDUAL DISEASE AND TREATMENT EFFICACY IN MULTIPLE MYELOMA

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Flow cytometry immunophenotyping (IF) has been shown to be a powerful technique for the discrimination between malignant and normal plasma cell (PC). In this study we utilised a panel of monoclonal antibodies to evaluate the sensitivity and specificity of six colour flow cytometry IF in detecting malignant PC in bone marrow samples from Multiple Mieloma (MM) patients at diagnosis (n = 35) and after I (n = 23) and II (n = 28) autologous stem cell transplantation (ASCT). To increase the sensitivity level of the technique we utilized a live gate acquisition strategy in which up to $1 \times 10^4$ CD138 positive PC cells were acquired. The PC aberrant phenotype identified at diagnosis was used as patient specific molecular asset to follow up analysis. CD56 was the most frequent aberrant marker observed at diagnosis (73% of cases), compared to CD117 (40%), CD28 (36%) CD20 (23%), CD33 (10%) and CD19 (0%) respectively. CD45 was dim or negative in 73% of cases. The proportion of CD138/CD56 positive / CD19 negative / clg Kappa or Lambda light chain restriction was the best immunological combination in identifying minimal residual disease after treatment. A CD28 strong expression was detected in the 3 cases with CSF infiltration and in 2 cases with plasmoblastic morpholoy. The II ASCT provide a greater reduction in the level of residual tumour PC in the majority of cases. Despite the low percentage of infiltrating PC after treatment (4%, range 1–10), the immunological discrimination between malignant and normal PC was possible in 68% of samples, with 87% flow cytometry positive cases that where negative by conventional cytology. We conclude that flow cytometry is a powerful technique in monitoring MM, particularly useful in low PC infiltration.

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FLUORESCENCE IN SITU HYBRIDIZATION DETECTION OF GENOMIC ABERRATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Fluorescence in situ hybridization (FISH) has improved the detection of genomic aberrations in patients with chronic lymphocytic leukemia (CLL). Using this tecnique, Döhner et al. (NEJM 2000) analyzed a large cohort of CLL patients showing that genomic aberrations are important independent predictors of disease progression and survival.

We report on 89 patients with CLL (mean age 62 years; range 35–83 years; 52 male and 37 female) for which FISH was used at diagnosis to detect the presence of 12q trisomy, 13q deletion, 11q deletion, and 17p deletion. Chromosomal aberrations were detected in 40 (46%) patients. Of them, 15 (38%) patients had 13q deletion, 7 (17%) patients trisomy 12, 7 (17%) patients 11q deletion, 6 (15%) 17p deletion, and 5 (13%) various abnormalities (2 patients 11q and 17p deletion; 1 patient 13q and 17p deletion; 1 patient 13q and 11q deletion; 1 patient 13q deletion and trisomy 12). Patients were then stratified in six groups according to the presence or absence of chromosomal aberrations (no abnormalities; trisomy 12; 13q deletion; 11q deletion; 17p deletion; various abnormalities) and clinical and biological features of each group were analyzed. No differences were found among the six groups of patients with respect to age, gender, Binet clinical stage, percentage of bone marrow infiltration, hemoglobin levels and platelet count at diagnosis. However, in patients carrying more than one abnrmality a greater number of peripheral blood lymphocytes was found. ZAP-70 and CD38 expression, and IgVH unmutated genes were also detected in patients with 11q and 17p deletions. Finally, no difference was found with respect to the need to therapy and the time to treatment. In conclusion, our preliminary data also confirm the negative prognostic significance of the presence of 11q and 17 p deletion as genomic aberrations detected at diagnosis in CLL patients.

## POLYCLONAL CD5-POSITIVE B-CELL EXPANSION IN A PATIENT WITH REUMATHOID ARTHRITIS (RA) AND ACUTE MYELOID LEUKEMIA (AML)

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The expansion of a subset of CD5-positive B-cells is commonly found in several autoimmune disorders. Flow cytometry (k:l light chains ratio) and/or molecular (V-D-J sequences) studies appear to be crucial in order to identify the monoclonal or polyclonal nature of cells. We report on a 70-year old woman, with a history of diabetes mellitus and RA, presented with pancytopenia (Hgb 7.0 g/dl; WBC 2,100/μl, 81% of which lymphocytes, Plts 79,000/μl). Physical examination showed only pallor. On peripheral blood film, no immature cells but only leucopenia with mature appearing lymphocytes and some dysplastic neutrophils were seen. The flow cytometric analysis showed predominance of T-cells (68%) and expansion of CD5-positive B-cells (20%), with normal k:l ratio. In bone marrow was found a remarkable infiltration (55%) of myeloid leukemic cells (CD13, CD33, CD117, MPO-positive) and a diagnosis of AML FAB M1 was made. Bone marrow lymphoid population (16%) showed 74% of T-cells, while the majority of B-cells were found to express CD5, with normal k:l ratio. Polyclonality of B-cells was also demonstrated by means of the molecular detection of V-D-J sequences. Two populations of B-cells (B1 and B2) have been recognized. B1-cells, a minor subset in the adult, are identified by the expression of CD5 which is also found on all T-lymphocytes. An important feature of B1 cells is the production of low affinity polyreactive Igs and seem to play an important role in autoimmune diseases such as RA.

## UTILITY OF FINE NEEDLE CYTOLOGY (FNC) AND FLOW CYTOMETRY IMMUNOPHENOTYPING (FCI) TO DIAGNOSE LYMPHOID DISORDERS

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The role of cytology to diagnose and classify non-Hodgkin's lymphoma (NHL) is controversial. We have evaluated the diagnostic role of the combined application of FNC and FCI in 30 patients with a suspected diagnosis of lymphoma. In 26 cases the patients presented with palpable masses (7 cervical, 2 supraclavicular, 1 inguinal, 10 axillary, 1 breast, 1 palatine tonsil, 2 parotid) and FNC was performed without any radiological guidance. In 4 cases FNC was performed on deep-seated masses (2 abdominal and 2 mediastinum) under ultrasound and CT guidance. In all but 3 cases (2 reactive hyperplasias and 1 lymphoblastic T-cell lymphoma) node biopsy was subsequently performed. Smears were stained with Diff Quik and Papanicolaou stain. The following MoAbs were used: CD45, CD2, CD3, CD5, CD4, CD8, CD7, CD10, CD19, CD20, CD22, CD23, k and l. In selected cases immunocytochemistry was also performed (CD45, CD30, CD15, EMA, cyclin D1, ALK-1). The series comprised 6 cases of Hodgkin's disease, 2 reactive hyperplasia and 22 NHL. Histopathology showed a high concordance with FNAC/FCI combined diagnosis. FNAC combined with FCI may allow a rapid discrimination between reactive hyperplasia and lymphoproliferative disorders. These techniques may be helpful in accurately subclassifying certain NHL, such as FL and MCL, for which a specific therapeutic approach is required.

## ZAP-70 AND CD38 EXPRESSION IDENTIFIES PROGNOSTIC SUBGROUPS OF B-CELL CHRONIC LYMPHOCYTIC (CLL) LEUKEMIA PATIENTS

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Several prognostic factors have been evaluated in order to predict an indolent or aggressive clinical course of B-CLL. We evaluated the simultaneous expression of ZAP-70 and CD38 in 157 pts (median age 64 yrs) with previously untreated CLL. 57 (36%) were positive for ZAP-70 and 46 pts (29%) were positive for CD38. Both ZAP-70 and CD38 expression were shown to independently predict the clinical course of the disease and were also found highly correlated to each other. Pts were then divided into 3 groups according to the simultaneous evaluation of ZAP-70 and CD38. In 81 pts (52%) there was a negative concordance of both molecules ($ZAP\text{-}70^-/CD38^-$); in 27 pts (17%) there was a positive concordance ($ZAP\text{-}70^+/CD38^+$); in 49 pts (31%) there was a discordant ZAP-70/CD38 expression. A comparison of the clinical and laboratory data of the 3 groups showed that in $ZAP\text{-}70^+/CD38^+$ pts higher bone marrow and peripheral blood lymphocytosis, lower Hgb levels, more advanced clinical stage, and higher number of unmutated $IgV_H$ status were found. $ZAP\text{-}70^-/CD38^-$ pts displayed a much shorter treatment-free interval [median 12 months vs 42 months in discordant pts and not reached in ZAP-70-CD38- pts ($p < 0.0001$)]. This data suggests that a simultaneous assessment of both $ZAP\text{-}70^-$ and $CD38^-$ need to be performed in all CLL cases to better identify prognostic sub-goups.

## UMBILICAL CORD AS SOURCE OF ADULT MESEN-CHYMAL STEM CELLS (MSC)

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Adult MSC are prevalently located within the stromal component of bone marrow. These cells show high self-

renewal potential and may differentiate into multiple cell lineages. This study was aimed to investigate the umbilical cord as potential source of MSC to be differentiated to osteoblasts (OB). MSC were derived from two cords and characterized by flow cytometry for both mesenchymal and hematopoietic markers. Cell morphology was analyzed in phase-contrast and fluorescence microscopy using FITC-Phallotoxin. We observed in culture a monolayer of fusiform or flat shape cells with a typical stressfiber pattern, showing the majority of mesenchymal markers as CD90, CD105, CD44 and CD13 with no expression of both hematopoietic and HLA-DR antigens. Therefore, MSC were induced to differentiate to OBs or adipocytes with dedicated media. After three weeks, the differentiation to OB or adipocyte lineage was assessed in relation to the expression of alkaline phosphatase or oil red O respectively. In addition, the expression of Runx2, BMP-2 and Osterix as transcription factors of OBs was detected by RT-PCR and confirmed the plasticity of these adult MSC. Our data support the derivation of MSC from umbilical cord for future application in tissue engineering of the bone.

## CD52 ANTIGEN EXPRESSION AND GENE PROFILE IN LYMPHOPROLIFERATIVE DISORDERS: THERAPEUTIC IMPLICATIONS

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CD52 is a glycosyl-phosphatidylinositol-anchored protein expressed on the surface of lymphoid cells. The anti-CD52 monoclonal antibody represents a therapeutic tool for patients with acute and chronic lymphoproliferative disorders. In the present study, we analyzed and quantified the CD52 expression on normal B and T cells, as well as on acute and chronic leukemias. The levels of CD52 gene expression were also evaluated.

In 20 normal donors, CD19+/CD20+ peripheral B lymphocytes expressed 9,581 CD52 molecules/cell (median value, range 6,234–26,078). T cells had significantly ($p = 0.0001$) lower levels of CD52 expression: 5,803 molecules/cell on CD3+/CD7+ cells (range 2,917–11,056). Interestingly, the CD4+ population expressed a significantly higher ($p < 0.0001$) number of CD52 molecules/cell than CD8+ lymphocytes (7,531 median value, range 4,340–12,092 vs 3,178, 2,204–7,591).

All neoplastic cells obtained from 191 chronic lymphocytic leukemia (CLL) patients, evaluated at diagnosis, expressed the CD52 antigen. The median number of molecules/cell was 12,848 (range 3,154–41,734). No difference in CD52 expression was documented between CD3+, CD4+ and CD8+ cells from CLL patients and normal controls.

In acute lymphoblastic leukemia (ALL), the CD52 antigen was found in 54% ± 34 of B-lineage ALL cells (113 cases) and in 39% ± 34 of T-ALL cells (39 cases): this difference was significant ($p < 0.02$). The levels of expression were lower than in normal B and T cells. Overall, 22.1% of B-lineage ALL were CD52 negative; in particular, all cases carrying the t(4;11) translocation failed to express the CD52 antigen. Within T-ALL, 38.5% of cases showed no CD52 expression with no correlation with the immunophenotypic and/or molecular profile. In all cases studied, a complete concordance was observed between the CD52 immunophenotypic picture and the gene expression profile.

## FLOW CYTOMETRIC MINIMAL RESIDUAL DISEASE EVALUATION IN ACUTE LYMPHOBLASTIC LEUKEMIA: A MULTI-CENTRIC STANDARDIZATION PROGRAM RESULTS OF THE AIEOP-BFM-ALL-FCM MRD-STUDY GROUP

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Single-laboratory experience showed that flow cytometric (FCM) assessment of minimal residual disease (MRD) in acute lymphoblastic leukemia (ALL) is feasible in most patients and provides independent prognostic information. It is, however, not known whether FCM analysis can reliably be standardized for multi-centric application. An extensive standardization program was installed in 4 collaborating laboratories which study FCM-MRD in children treated with the AIEOP-BFM-ALL 2000 protocol. This included methodological alignment, continuous quality monitoring, as well as personnel education. Blinded interlaboratory tests of list-mode data interpretation concordance (n = 202 blood and bone marrow samples from follow-up during induction of 31 randomly showed a very high degree of of a total series of n = 395) showed a very high degree of inter-rater agreement among the 4 centers despite differences in cytometers and software usage (intra-class correlation coefficient 0.979 based on n = 800 single values). Comparing the data from sample exchange experiments and from the independent patient cohorts from the 4 centers (regarding positive samples per time-point and type of material, as well as risk estimates) differences were amounts of MRD below 0.1%. In conclusion, MRD-evaluation by FCM in ALL can be standardized for reliable multi-centric assessment in large trials.

## FLOW CYTOMETRY ANALYSIS OF RETICULATED PLATELETS: A NEW-SENSITIVE TEST FOR THE DETECTION OF RIBONUCLEIC ACIDS CONTENT BY AN ANTI-BROMODEOXYURIDINE MONOCLONAL ANTIBODY

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INTRODUCTION: Current strategies for the quantitation of ribonucleic acids in reticulated platelets are assessed with fluorescent dyes as thiazole orange and flow cytometry. Nevertheless, standardized operation procedure has not yet been achieved. Our goal is to develop a new standardized flow cytometry assay by using anti-bromodeoxyuridine monoclonal antibody, with an unexpected cross-reaction with ribonucleic acids. MATERIALS AND METHODS: Peripheral blood samples obtained from 150 healthy donors and from 120 autologous peripheral blood stem cell transplanted were drawn into a vacutainer-EDTA tube and freshly platelet rich plasma was then collected. Platelets were identified by FITC-CD41 a surface staining and their ribonucleic acids content measured on paraformaldehyde fixed platelets by intracytoplasmic staining with specific anti-bromodeoxyuridine monoclonal antibody plus PE-F(ab′)2 fragments of rabbit anti-mouse lg. Isotype controls were incubated with mouse IgG1. The double stained samples were analyzed on a FACScan flow cytometer. RESULTS: Reticulated platelets were CD41a/ anti-BrdU positive events on all samples tested. A significant correlation was found between percentage and mean fluorescence intensity of reticulated platelets ($r = 0.85$, $<0.01$). The mean percentage was statistically higher in women ($5.60 \pm 2.8\%$, $p < 0.01$) than in men ($3.9 \pm 2.0\%$). In addition, the mean percentage and absolute counts of reticulated platelets in healthy individuals and patients were ($4.6 \pm 3.1\%$; $10.6 \pm 10.0 \times 10^3/\mu l$) and ($5.7 \pm 2.6\%$; $5.3 \pm 4.1 \times 10^3/\mu l$), respectively. A high correlation was found in platelets donors ($r = 0.82$, $p < 0.01$), in contrast to patients were no relationship was observed. CONCLUSION: We present an alternative robust, accurate, time efficient assay for the detection of reticulated platelets, that combining the attributes of flow cytometry and the monoclonal antibody strategy is capable of achieving standardisation across clinical laboratories.

## MINIMAL RESIDUAL DISEASE (MRD) DETECTION STRATEGY IN ACUTE MYELOID LEUKEMIA (AML). A SIX-COLOR STUDY ON 27 CONSECUTIVE CASES

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Flow cytometry (FCM) has emerged as the one of the most promising methods for detecting submicroscopic populations of acute leukemia blast cells. FCM detection of MRD is based on the identification of antigen combinations expressed on leukemic cells (but not on normal cells) with a sensitivity of $10^{-4}$. We applied an antibody panel including 22 five-color combinations for the diagnosis of AML. The exact identification of blast cells allowed us to define the ideal six-color combinations for MRD monitoring. We focused on two particular aspects of leukemic immunophenotype: i) mixed-lineage (myeloid+lymphoid) profiles and ii) maturation asynchronisms (early+late specificities). We studied, during the last 7 months, 27 consecutive cases with AML. We observed 4 cases (15%) showing mixed-lineage features only. The presence of lymphoid markers (CD5, CD2, CD7, CD19, CD56) and myeloid markers (CD13, CD33) associated to CD34 suggested the combination CD34/ CD7/ CD45/ CD33/ CD13/ CD14. CD7 could be substituted by another lymphoid antigen expressed on leukemic cells. In 12 cases (44%) blast cells were characterized by maturation asynchronisms. The expression of CD34 and CD117 (progenitor-associated antigens) associated to the presence of CD11b, CD11c, CD14, CD10 or CD24 (late myeloid markers), allowed us to use combinations such as CD34/ CD117/ CD45/ CD33/ CD13/ CD14 and CD24/ CD34/ CD45/ CD33/ CD10/ HLA-DR. In 11 cases (41%) blast cells with simultaneous mixed-lineage and maturation asynchronism were detected. Combinations for these cases included markers identifying both defects, e.g CD7/ CD11c/ CD45/ CD34/ CD13/ CD14. To the best of our knowledge, this is the first study in which in six-color strategy to detect MRD was used on a routine basis and the definition of an effective MRD strategy in 100% of cases of AML was demonstrated.

## FLT3 PHOSPHORYLATION STATUS AND ITS RESPONSE TO DRUGS CAN BE MONITORED BY FLOW CYTOMETRY IN AML BLAST CELLS IN VITRO AND IN VIVO

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FLT3 mutation and overexpression in most acute myeloid leukaemia patients make this tyrosine kinase receptor an attractive therapeutic target. FLT3 kinase inhibitors are actually in clinical trials, thus it is critical to develop a reproducible and standardized method for screening of FLT3 receptor activation and for monitoring its inhibition in response to drug in AML patients.

We analyzed the expression level of FLT3 receptor (CD135) by FACS analysis. We developed a flow cytometry method to analyze phosphorylated FLT3 (P-FLT3) in samples with $<10^5$ cells. The method was first validated in FLT3 wild-type (HL60/WT) and mutant (MV4-11/ITD$^+$) as well as FLT3 negative (K562) cell lines. The method also proved to be reproducible in AML patient samples.

Analysis was performed after exposure to drugs (CEP-701 and SU11657), in vitro and in vivo. In response to

increasing drug concentrations, there was a linear reduction in P-FLT3. Intracellular flow cytometry analyses correlated with western blot and XTT assays. Flow cytometry data also correlated with *FLT3* mutational status.

The results validate a rapid method to detect P-FLT3 protein at the single cell level by flow cytometry, and enable an accurate assessment of FLT3 kinase activity in blast cells in response to novel tyrosine kinase inhibitors.

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HUMAN PLACENTA: AN ALTERNATIVE SOURCE OF MESENCHYMAL STEM CELLS

**Manetta T.,[1] Rustichelli D.,[1] Mareschi K.,[1] Ferrero I.,[1] De Fazio R.,[2] Anselmino A.,[1] Morterra G.,[1] Gunetti M.,[1] and Fagioli F.[1]**

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Background: Human placenta represents an alternative source of stem cells endowed of high plasticity. Our purpose was to set up a method for mesenchymal stem cells (MSCs) isolation and expansion and to evaluate their immunophenotype characteristics and differentiation capacity. Methods: Term placenta tissue was harvested after informed consent from women undergoing elective caesarean section. Tissue specimens from approximately 1 $cm^3$ were digested in Phosphate Buffer Solution (PBS) supplemented with penicillin, streptomycin, clarithromycin, collagenasis and dispasis for 1 hour at 37°C. The enzymatic reaction was stopped by adding 10 volumes of α-Mem with 10% Fetal Bovine Serum. The single-cell suspension was cultured in α-Mem with 10% Fetal Bovine Serum and detached weekly. Cellular growth, immunophenotype and differentiative potential were evaluated during the in vitro expansion. Results: Five primary cellular cultures were obtained from 6 samples. The cells obtained had fibroblastic aspects with a high proliferative potential and were positive for CD90, CD105, CD29, CD44, CD73. Cumulative population doubling (PD) after 5 passages was 3.6 ± 2.3 for the MSCs isolated from bone marrow of healthy donors and 17.1 ± 1.7 for the MSCs isolated from the placenta suggesting an enhanced cellular growth. The MSCs also differentiated in osteoblasts, adypocytes and chondrocytes. Conclusions: All these data suggest that human placenta is an abundant source of MSCs easy to isolate and expand, having a higher proliferative potential compared to the bone marrow-derived MSCs.

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MESENCHYMAL STEM CELLS ISOLATED FROM AMNIOTIC FLUID DIFFERENTIATE IN NEURAL CELLS

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Introduction: Amniotic fluid (AF) contains stem cells which, due to their ontogenetic origin, have a high proliferative and differentiative potential. The aim of our work was to set up a method for mesenchymal stem cells (MSCs) isolation and expansion and to evaluate their immunophenotype characteristics and differentiation capacity. Methods: AF was harvested from women undergoing aminocentesis for routine prenatal diagnosis at 14–16 weeks of pregnancy. AF samples were centrifuged and the resulting pellets were plated?-Mem with 10% Fetal Bovine Serum. Cellular growth, immunophenotype, stemness markers, telomere length and differentiative potential were evaluated during the in vitro expansion. Neural Progenitor Maintenance Medium (NPMM, Lonza) was used for neural induction. Results: Twenty six primary cellular cultures were obtained from 28 samples. The cells obtained from the samples containing more than 6 ml of AF, had fibroblastic aspects with a high proliferative potential and were positive for CD90, CD105, CD29, CD44, CD166. The expansion did not induce a significant telomere length shortening and the molecular analysis showed the expression of Oct-4, Nanog and Rex-1. Moreover, the MSCs isolated from AF differentiated in osteoblasts, adypocytes and chondrocytes. The cells cultured in NPMM expressed neural markers and functionally active voltage-dependent $Na^+$ and $Ca^{2+}$ channels. Conclusion: All these data suggested that AF is an important multipotent stem cell source with a high proliferative potential able to transdifferentiate in cells with neuronal characteristics.

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CIRCULATING ENDOTHELIAL CELLS (CECs) AND ENDOTHELIAL PROGENITOR CELLS (CEPs) IN PERIPHERAL BLOOD (PB) FLOW CYTOMETRIC DETECTION IN HEALTHY SUBJECTS (HS)

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Background: CECs play an important role in neovascularisation and tumor growth. CECs, resting (rCECs) and activated (aCECs), with CEPs are currently evaluated as potential biomarkers of anti-angiogenic therapy. This is important to correlate the level of CECs with disease status, known prognostic factors and response to treatment. As preliminary phase of a clinical study, we wanted to quantify, CECs and CEPs values of HS that will be utilized as controls vs cancer pts treated with antiangiogenetic drug. Methods: Samples of PB from 50 HC (M/F:25/25, median age 40 yrs) were analysed. A panel of MoAbs, including anti-CD45 to exclude hematotoietic cells, anti-CD34, -CD106, -CD133, -CD146, and appropriate analysis gates were used to enumerate rCEC, aCEC and CEPs. % cells numbers were calculated after acquisition of at least 100.000 cells per PB sample. Six parameter, 4-color FCM procedures were perfomed with a BD FACS Canto. We defined rCECs as CD45−, CD146+ CD34+, CD106− aCECs as CD45−, CD34+, CD146+, CD106+ and CEPs as CD45−, CD146+, CD34+ and CD133+. Results: Our % mean values of rCECs and aCECs in peripheral mononuclear cells were 0.004% and 0.006% respectively; mean value of CEPs was 0.001%. Conclusions: CECs and CEPs are

rare events in PB. The FCM is a accurate and sensitive method for detection and quantification of CECs and CEP and it can be utilized in the clinical setting as to monitor tumor response during treament with antiangiogenetic drugs. In view of the planning of cancer anti-angiogenic trials is necessary identify and enumerate CECs and CEPs in a significant group of HS.

## MULTIPARAMETER FLOWCYTOMETRY OF CEREBROSPINAL FLUID INVOLVED BY LEUKEMIA/LYPHOMA CELLS

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Multiparameter flow cytometric analysis of cerebrospinal fluid (CF) is useful to evaluate leukemia/lymphoma patients with central nervous system (CNS) involvement of the disease. In 24 specimens of 22 patients (11 male and 11 female; median age 35 yrs; range 13–58 yrs) with different types of leukemia/lymphoma (2 AML; 3 T-ALL; 8 B-ALL CD10+; 9 DLBCL) the presence of pathological clone in CF has been confirmed or excluded by means of flow cytometry. The panel of MoAbs used was defined according to those of bone marrow or peripheral blood of diagnosis and final histologic diagnosis of lymphoma. CD45 (common leukocyte antigen) gating strategy was used to define cell population of interest. In 15 cases (3 T-ALL; 6 B-ALL and 6 BLBCL) a pathological clone was found. In the remaining 9 cases a CNS involvement was ruled out by means of flow cytometric analysis. In all cases a concordance with CF citology was also found and only in 1 case (DLBCL) CNS involvement was evidenced by means of imaging technique (MRI).

Our results suggest that flow cytometric analysis of CF is a useful indicator of malignancy and reflects leptomeningeal involvement. Furthermore, it appears as a reliable and quick technique able to recognize occult leptomeningeal disease also in absence of signs and symptoms of disease.

## CLONAL EVOLUTION OF CMRL-T CELL LINE ASSESSED BY TEN COLOR FLOW CYTOMETRY

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Recently, we were able to accomplish the stabilization of a new hematopoietic cell line, named CMRL-T, derived from the peripheral blood of a 13y old Italian girl affected by T-cell Acute Lymphoblastic Leukemia (T-ALL) at relapse. CMRL-T shows a unique phenotype characterized by asynchronous maturation program, with the contemporary expression of CD34+, TdT and TCRαβ molecules, as well as by two different TCR-beta chains (Vβ3 and Vβ12), an unusual feature described only for a rare T-cell subset present in normal peripheral blood. Karyotype analysis disclosed unique features of CMRL-T cells, i.e. t(3;17) (p14;p11) and t(4;11) (p16;q13) chromosomal alterations. In this work we compared the CMRL-T antigenic pattern at the moment they started to grow in vitro (1999) to their immunophenotype at the end of in vitro stabilization (2006), by the use of FACSAria and ten color multidimensional flow cytometry to the aim of describing the clonal evolution of CMRL-T cells. CMRL-T primary culture was composed by a heterogeneous cell population as evidenced by the contemporary presence of 3 different subpopulations defined as: 1) CD34+, CD45dim, CD3dim, TCRαβdim, CD4−, CD8− 2) CD34−, CD45dim, CD3dim, TCRαβdim, CD4−, CD8− 3) CD34−, CD45+, CD3+, TCRαβ+, CD4+, CD8+. Immunophenotyping of CMRL-T after the complete stabilization displayed a homogeneous population exhibiting the features of the subset with the most immature phenotypic pattern (population N°1). We propose multidimensional flow cytometry as a suitable method to depict clonal evolution of newly established cell lines. Moreover, we propose this cell line as a useful mode not only to study the leukemic processes involved in immature T-ALL but also the molecular and cellular mechanisms implicated in T-cell maturation and inβ-chain selection during thymic ontogeny.

## SIX-COLORS FLOW CYTOMETRY ANALYSIS OF CD34+ HEMATOPOIETIC STEM CELLS IN CRYOPRESERVED EARLY PRE-TERM HUMAN CORD BLOOD SAMPLES

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During the last decades, term cord blood (T-CB) hematopoietic cell populations have been extensively characterized due to their usefulness for the care of severe hematological malignancies in pediatric patients. However, less information is available on early pre-term cord blood (EPT-CB) hematopoietic stem cells. The objective of our study was to depict the antigenic mosaic of CD34+ stem cell population present in EPT-CB liquid nitrogen cryopreserved samples (8 cases) by the use of BD FACSCanto II and six-color flow cytometry analysis. T-CB samples (3 cases) were used as controls. FACS-Diva data analysis showed that the CD23+ cell compartment was larger in EPT-CB when compared to T-CB, being of 1.73% and 0.25%, respectively ($p < 0.01$). Successively, the CD23+ hematopoietic stem cell population was screened for a series of activation structures, adhesion molecules, cytokine-receptors and efflux-related proteins, such as CD38 (ADP- ribosyl-cyclase), CD29 (β1-integrin), CD31 (PECAM-1), CD90 (Thy-1) CD117 (c-kit), CD135 (Flt3) and CD243 (MDR1). CD34+/CD38- stem cell compartment significantly decreased parallel to the progress of gestational age, being of 11.1%, 7.5%, 4.35% and 1.53% at $17^{th}$, $21^{th}$ $27^{th}$ $40^{th}$ week, respectively ($p < 0.01$). CD29 and CD31 were highly expressed in both EPT-CB and T-CB samples, while CD135 was negative in all

cases. CD90 was expressed at higher extent in EPT-CB cases as compared to T-CBs ($p < 0.01$). By contrast, CD117 and CD243 were highly displayed by T-CB stem cells as compared to EPT-CB CD34+ cells. To the best of our knowledge, this is the first report proposing an extended multidimensional analysis of CD34+ hematopoietic compartment in EPT-CB samples. Our data suggest that EPT-CB specimens, currently drawn for prenatal karyotypic diagnosis in fetuses with critical alterations, may be a potential source of highly undifferentiated hematopoietic stem cells useful for planning future cell therapy studies.

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UNDERDIAGNOSED SYSTEMIC MASTOCYTOSIS: THE ROLE OF FLOW CYTOMETRY

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The prevalence of Systemic Mastocygtosis (SM) is difficult to assess due to the possible absence of skin lesions and/or specific signs and symptoms.

Aim of our study was to evaluate the role of flow cytometry (FC) in the identification of SM patients in comparison to other techniques.

We studied 47 adult patients with suspected SM. Among them, 16 cases had been diagnosed as cutaneous mastocytosis (CM), 27 had presented severe anaphylactic reactions after hymenoptera stings in association with high basal serum tryptase levels, and 4 patients had unspecific signs and symptoms.

According to international consensus for diagnosis of SM, each patient was evaluated as follows: basal tryptase serum level, bone marrow (BM) aspirate and BM biopsy (anti-tryptase monoclonal antibody staining). The presence of mast cells (MCs) on BM aspirate was investigated using a specific five-color monoclonal antibody combination (CD25/CD2/CD45/CD34/CD117). In addition, we assessed the presence of D816V KIT mutation in BM mononuclear cells by restriction fragment length polymorphism analysis.

Based on clinical, laboratory, immunophenotypic and molecular findings the definitive diagnosis was reached in 43.47 patients (32 SM, 8 Monoclonal MCs Activation Syndrome,and 3 CM).

By using FC we were able to identify cells with the SM features (i.e.CD117++/CD34−/CD25+ or CD2+) infiltrating the BM i 37/40 cases (93%). In contrast, 78% of cases resulted positive by molecular biology analysis and 61% by BM histology. In addition, FC analysis could detect MCs expressing aberrant phenotypes in the presence of low BM-infiltrating SM MCs (median 0.12% of CD45+ cells; range 0.002%–1.46%).

Therefore, FC shows a good efficiency in the identification of abnormal MCs compared with other techniques and could represent the tool of choice to diagnose patients with suspected SM.

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BIOLOGICAL FLUID EXAMINATION IN HEMATOLOGIC MALIGNANCIES: A COMPARISON BETWEEN FLOW CYTOMETRY AND CYTOLOGY

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The role of immunophenotyping in detecting malignant cell contamination of biological fluids is largely unexplored.In order to evaluate whether a correspondence exists between flow cytometric findings and morphological findings on cytospin slides, 53 biological fluid samples [bronchoalveolar lavage (BAL), n = 5; ascitic fluid (AF), n = 2; scrotal fluid (SF), n = 2; pleural effusion (PE), n = 10; and cephalorachidian fluid (CRF), n = 34] drawn between 2002 and May 2007 from patients with hematologic neoplasms were retrospectively analyzed. The samples represented 8.6% of the biological fluid samples analyzed during the same period. Diagnoses were as follows: chronic myelomonocitic leukaemia (CMML) (n = 1), FAB M3 acute myeloid leukaemia (AML) (n = 9), FAB M3 acute myeloid leukaemia (CLL) (n = 4), follicular lymphoma (FL) (n = 1), Hodgkin disease (HD) (n = 1), acute lymphoblastic leukaemia (ALL) (n = 7), lymphoblastic lymphoma (LBL) (n = 2), chronic myeloid leukaemia lymphoid blastic crisis (CML-LBC) (n = 4), high grade non-Hodgkin lymphoma (NHL), Burkitt-like (n = 6), diffuse large B-cell lymphoma (n = 6), peripheral T-cell lymphoma (n = 6), pleural effusion lymphoma (PEL) (n = 1), and NHL, unspecified (n = 4).

Samples were stained using the pan-leukocyte marker (CD45) and/or with multicolor panels (FITC/PE/PerCP/APC) specific for the hematologic disease. Flow-cytometric data from 51 samples (96%) could be retrospectively compared with morphological findings obtained from cytospin slides. Among the 27 cases in which flow cytometry detected neoplastic cell contamination, the latter was observed in only 18 cases by morphologic exam, possible presence (n = 1) or absence (n = 8) of neopastic cells being observed in the remaining. In one case, in which neoplastic cells in the AF of a CMML was suspected but not confirmed (atypical cells could not be otherwise specified), flow cytometry disclosed the presence of 53% $CD45^+$ cells displaying a $CD13^+CD14^+CD64^+CD34^-CD117^-$ phenotype. Three morphologically negative CRF displayed minimal residual disease by disease-specific panels (0.21% cells in CML-LBC, 1% cells in B-CLL, and 0.26% cells in peripheral T-cell lymphoma). The other 5 morphologically negative cases were 2 PE and 3 CRF from CLL (n = 2), LBL (n = 1), high grade NHL, Burkitt-like (n = 1), and peripheral T-cell lymhoma (n = 1), displaying 10% to 99% neoplastic cell contamination as detected by flow cytometry.

Among the 24 cases in which flow cytometry did not detect neoplastic cell contamination, the latter was not

observed in only 18 cases by morphologic exam, possible (n = 2) or sure (n = 4) presence of neoplastic cells being observed in the remaining ones. In three cases (PE: n = 1), AF: n = 1, CRF: n = 1), morphologic suggestion of diffuse large B-cell lymphoma contamination was not confirmed, since lymphocytes were clearly negative for disease-speciffic markers. Further two morphologically positive CRF from M3 AML did not show $CD45^+$ cells by flow-cytometry. The only case in which a false negative result was obtained by flow-cytometry ($CD45^+$ cells did not stain with lymphocyte markers) was the PEL, diagnosis being performed o the basis of immunohistochemcial detection of human HHV-8 on PE citospin slide.

Our data suggest that sensitivity and accuracy of flow cytometry in detecting neoplastic cells in biological fludis of hematologic patients is higher than those of morphologic evaluation.

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PODOSOME FORMATION AND CD11b/CD18 EXPRESSION IN LEUKEMIC THP-1 MONOCYTES PROTECT DIFFERENTIATING ADHERENT CELL SUBSET AGAINST APOPTOSIS INDUCED BY CYTO- SKELETION-UNAFFECTING AGENTS

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Phorbol ester PMA (6-60 nM) induces a gradual macrophagic differentiation of THP-1 monocytes correlated with shape, adhesion and motility changes. Agents unaffecting (Actinomycin D: Act D) and affecting (Vinblastine: VBL) cytoskeleton induce apoptosis in low differentiated or differentiated cells, respectively. Less adherent (low/mid differentiated) cells are sensitive to Act D apoptogenic stimuli, while adherent differentiated macrophages are apoptosis-protected. This can be related to the appearance of adhesive actin structures with podosome features, evident at the cell periphery and in pseudopodia of typical and fibroblastoid macrophages. Morpho-cytometric analysis shows a relation between podosome actin labelling and $\beta_2$ integrin CD11b/CD18 immunoreaction. The resistance against Act D of macrophagic differentiated cells may be a consequence of integrin-mediated survival signals, during cell-substrate anchorage. Then, interaction of microfilaments and microtubules with podosomes could play a role in protecting against cell death: these adhesive structures can transmit signals from the extracellular environment to cytokeleton. On the contrary, microtubule affecting stimuli (VBL treatment) delete the resistance in more adherent differentiated cells. Microtubules display more persistent association with podosomes, as compared with the transient interactions shown for typcial focal adhesions. Then, VBL-induced microtubule depolymerization could determine the loss of contacts with the substrate and consequently cell detachement and death by anoikis. Our findings indicate that apoptotic effects are strictly dependent on the differentiation status: apoptotic sensitivity appear to depend on the condition of culture growth as proliferating and non-adherent monocytes or as differentiated and adherent macrophages.

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ANALYSIS OF ENDOTHELIAL PROGENITOR CELLS (EPCs) IN THE PERIPHERAL BLOOD OF PREGNANT WOMEN

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Endothelial progenitor cells (EPCs) endowed with the potential to proliferate and differentiate into mature endothelial cells are present in adult peripheral blood and are though to contribute to the mainstenance of endothelial function and to sustain any request of angiogenesis, included endometrial repair after menstruation and neovascularization in the utero-placental circulation.

To assess whether EPCs are increased in pregnant omen, in the present study we enumerated EPCs in the peripheral blood during physiologic pregnancy. Among different antigenic profiles, EPCs were defined as CD34+/KDR+/CD133+ cells and quantified by flow cytometry directly in fresh blood samples. Mature circulating endothelial cells (CECs) identified as CD45 − /CD31+/CD145+ cells, were also analyzed. We observed that EPCs are markedly increased in pregnant women compared with age-matched controls (p = 0.02). Intriguingly, also CECs resulted increased during pregnancy. These results may suggest that pregnancy per se promotes the proliferation and/or the mobilization of EPCs from the bone marrow into circulation. Because the fine mechanisms governing angiogenesis have not yet been clearly elucidated our data will be related to plasmatic levels of angiogenic factors and sexual hormones.

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CHARACTERIZATION OF CELLULAR MODELS FOR THE STUDY OF MITOCHONDRIAL FERRITIN PHYSIOPATHOLOGICAL ROLE IN SIDEROBLASTIC ERYTHROPOIESIS

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We recently described a mitochondrial ferritin (MtF) in the erythroblasts of refractory anemia with ring sideroblasts (RARS) that may play an important role in regulating iron homeostasis and heme synthesis. Since preliminary findings indicate that MtF+ cells are more resistant to the apoptotic signals, we evaluated at which stage RARS erythroblasts express MtF and whether its accumulation reflects a defence mechanism in relation to apoptosis and proliferative activity. CD34+ marrow cells from 29 patients with myelodysplastic syndromes (MDS, 16 RARS and 13 RA) and from 8 healthy donors were cultured by a liquid culture procedure; samples were removed at (days 0, 4, 7, 11 and 14 for the evaluation of ferritins (MtF, HF, LF) and of Ki-67 antigen distribution using immunostaining and flow cytometry analysis. Apoptosis was measured by the translocation of cytocrome *c* (cyt *c*) to cytosol and by TUNEL technique. Flow cytometry analysis was performed using anti CD71,

anti Glycophorin A (GlyA), and anti CD34 antibodies, CD34+ cells of all samples were negative for MtF; while MtF was barely detectable in few cells from controls and RA patients, RARS samples showed an early expression of MtF continuously increasing (12– 18%, day 4–14). HF and LF levels were variable, tendentially higher in RA cases. Cyt *c* release was more pronounced in RARS and the apoptotic rate was higher in all MDS cases than in controls. Ki-67+ cells increased during culture in controls (26–63%, day 4–14), whereas they remained constant in MDS cases. A continuous increase of erythroid cells was observed (62% GlyA+ cells, day 14) and in MDS the GlyA+ cells showed a downregulation of CD71. Our findings show that the expression of MtF occurs very early in RARS erythroid differentiation; the aberrant expression of MtF, HF and CD71 in MDS confirms that MDS erythroid precursors have an iron-loaded phenotype associated with increased apoptosis and diminished proliferation, suggesting a close relationship between altered iron metabolism and MDS pathogenesis.

PRION-LIKE DOPPEL GENE (PRND): A NEW MOLECULAR MARKER POTENTIALLY INVOLVED IN LEUKEMOGENESIS

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The PRND gene encodes Doppel (Dpl), a protein that is strongly expressed in testis and at much lower levels in other tissues. The physiological role of this prion-like protein is unknown. Recently, we observed a weak Dpl expression in normal CD34+ bone marrow cells and high levels of Dpl in leukemic cell lines and in bone marrow cells from patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). In order to clarify the clinical and biological relevance of Dpl overexpression in these disorders, we searched for possible correlations among Dpl expression, biological parameters and clinical-pathological features. Moreover, we characterized PRND transcriptional and translation patterns. Immunocytochemistry, flow cytometry, biochemical and molecular studies were carried out on bone marrow cells from 64 AML patients, 98 MDS patients and 16 non-hemopathic subjects. Dpl, barely detectable in normal controls, was detected in almost all AML and MDS cases. No relationship was observed between Dpl levels and clinical or laboratory features nor did Dpl levels predict response to therapy in AML or disease progression in MDS. In AML cases achieving complete remission a significant reduction of both transcript and protein levels ($P = 0.02$) was observed. In 5 relapsing patients Dpl was overexpressed at levels similar to those observed at onset. Dpl behaviour was variable during MDS evolution towards AML. In pathological samples Dpl was abnormally localized in the cell cytoplasm. This ectopic localization was probably dependent on abnormal cellular trafficking because of glycosylation pattern modifications of the protein. Also an abnormal nuclear retention of the transcript was observed. Our findings confirm the clinical usefulness of Dpl evaluation for AML or MDS diagnosis and for the assessment of minimal residual disease. Studies are in progress to better understand which factors may contribute to the modulation of PRND activity.

## IMMUNOLOGY

ABLATION OF T-HELPER 1 CELL DERIVED CYTOKINES AND OF MONOCYTE-DERIVED TUMOR NECROSIS FACTOR-A IN HEREDITARY HEMORRHAGIC TELEANGIECTASIA: IMMUNOLOGICAL CONSEQUENCES AND CLINICAL CONSIDERATIONS

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Experimental evidences on the adaptive immune response in patients with hereditary hemorragic teleagiectasia (HHT) are lacking. Here, we report in 9 patients with HHT a multiple deficit involving the intracellular expression of T helper (h) 1-derived cytokines [Interferon (IFN)-γ, Interleukin (IL)-2 and Tumor Necrosis Factor (TNF)-α] and of monocyte-derived TNF-α. On the other hand, percentages of Th2-derived cytokines (IL-4, IL-5 and IL-10) were normal or, in some cases, above normality. Quite interestingly, monocyte-derived IL-10 was detectable in 5 out of 9 patients in a percentage of cells comparable to controls or exceeding normal levels. Taken together, these data point out, in HHT, an ablation of Th1-responses, while Th2-type cytokines are preserved, thus exerting either a suppressive effect on Th1-cells (via IL-4 and IL-10) or an antiinflammatory response on monocyte-derived TNF-a (via IL-10). Furthermore, monocyte-derived IL-10 may also contribute to the antiinflammatory activity seen in HHT. According to current literature even if patients with HHT do not exhibit certain diseases, such as autoimmune diseases, cancer and abnormal responses to pathogens, the observed immune deficits need to be diagnosed and therapeutically corrected.

NEUROPLIN-1 (Nrp1) EXPRESSION IDENTIFIES A NEW SUBSET OF $CD4^+CD25^{HIGH}FOXP3^+$ REGULATORY T CELLS (Treg) IN HUMAN LYMPH NODES (LN). MODULATION BY PREOPERATIVE CHEMORADIATION IN CERVICAL CANCER (CC)

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In LN, the semaphorin III receptor Nrp1 identifies a Treg population ($Nrp1^+$Treg) expressing markers of acti-

vated Treg, i.e., CD45RO, HLA-DR and GITR. $Nrp1^+$Treg proliferate poorly in vitro, and exert contact-dependent in vitro suppression of $CD4^+$ and $CD8^+$ T cell proliferation and cytokine secretion. In keeping with their activated phenotype, $Nrp1^+$Treg are more efficient than Nrp1-Treg at inducing suppression. Nrp1 is also expressed on a subset of $CD25^{int}$ and CD235-$CD4^+$T cells that contain comparatively lower levels of Foxp3 message and protein, GITR and CD45RO. In contrast, circulating $Nrp1^+CD4^+$T cells are a minor subset that does not express CD25 and is devoid of Foxp3 message and protein. In LN, Nrp1 may mediate homotypic interactions between $CD4^+$T cell and plasmacytoid dendritic cell, constitutively expressing Nrp1, thereby favouring the generation/activation of Treg. We previously showed that Treg may curb anti-tumour T cell response in CC. We show here that $Nrp1^+$Treg level drops in tumour draining LN of CC patients following preoperative chemoradiation therapy in direct relationship with the reduction of tumour mass, suggesting that $Nrp1^+$Treg elimination facilitates the generation of T cells mediating the destruction of neoplastic cells left behind after cytotoxic therapy.

## HUMAN MESENCHYMAL STEM CELLS PROMOTE SURVIVAL OF T CELLS IN A QUIESCENT STATE

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Mesenchymal stem cells (MSC) are part of the bone marrow where they support survival of haematopoietic stem cells. MSC modulate also the immune response as they inhibit proliferation of lymphocytes. To investigate whether MSC can support survival of cells we studied MSC capacity of rescuing T lymphocytes from cell death induced by different mechanisms. For such purpose several flow cytometric assays have been used. We observed that MSC prolong survival of unstimulated t cells and apoptosis-prone thymocytes cultured under starving condition. MSC rescued T cells from Activation Induced Cell Death (AICD) by downregulation of Fas and Fas L on T cell surface and inhibition of caspases involved in cell death. MSC weakened also Fas receptor mediated apoptosis of Jurkat leukemic T cells. In contrast, rescue from AICD was not associated with a significant change of Bcl-2. Accordingly, MSC showed a minimal capacity of rescuing Jurkat cells from chemically induced apoptosis disrupting the mitochondrial membrane potential regulated by Bcl-2. These results suggest that MSC interfere with the Fas receptor regulated process of apoptosis. Overall MSC can inhibit proliferation of activated T cells while supporting their survival in a quiescent state providing a model of their activity inside the HSC niche.

## CONTINUOUS POSITIVE AIRWAY PRESSURE (CPAP) MODIFIES PERIPHERAL BLOOD IMMUNOPHENOTYPE IN PATIENTS WITH OBSTRUCTIVE SLEEP APNEA SYNDROME (OSAS): PREDICTIVE VALUE OF THE CELLULAR PATTERNS

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OSAS is a respiratory disease due to atherosclerotic mechanisms; overnight CPAP treatment decreases apnea-hypopnea index (AHI). Aim of the study: to define if OSAS patients (OP) have a peculiar pattern of blood mononuclear cells and if it changes after one-night CPAP.

Pts and Methods: 57 OP (44 males and 13 females, AHI $>$ 20/hour) are studied before and after (T0-T1) overnight CPAP. By flowcytometry we determined CD4, CD8, activated-T, B, NK lymphocytes and DR+ and CD69+ monocytes. Results: T0 phenotype is normal At T1: all OP show significant increase of CD4+ lymphocytes and decrease of NK cells; the 38 OP with improvement (AHI$<$ 20) increase also CD3+CD69+, CD4+CD25+, CD20+ cells and up-regulated HLA DR on monocytes. OP whithout improvement (AHI$>$20) show at T0 more CD3+ NK+ and T-activated lymphocytes, while at T1 they increase activated monocytes (CD14+69+). Discussion: one-night-CPAP can modify the cellular pattern perhaps after down-regulation of adhesion molecules and mobilization of cells from the site of lesion. A sub-group of patients have a more aggressive pattern at T0 and don't improve AHI after one-night-CPAP.

## INTRATISSUTAL EFFECTS OF ANTI CD20 RITUXIMAB (RTX) IN A PATIENT WITH GRAVES' OPHTALMOPATHY.

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Graves' disease (GD) is a B-cell-mediated disease, characterized by thyroid associated ophtalmopathy (TAO). Aim of the study: to evaluate the clinical effect of RTX on GD and TAO and to determine the cellular patterns in 4 different tissue specimens: blood (PB), orbit (O), thyroid (THY) and neck lymphnode (LN) Methods: one patient with GD and TAO underwent RTX treatment and thyroidectomy after 7 month treatment. Endocrinological and ophtalmological evaluations were made. Cell analysis was made by flowcytometry (after homogenization of surgical specimens) and by immunohistochemistry. Results: RTX induced prolonged (6 months) peripheral B depletion and amelioration of TAO, but didn't affect hyperthiroidism. No lymphocyte was recovered from O specimen. Lymphocyte subpopulations in PB, THY and LN were

respectively: CD3+: 80 / 74 / 92%; NK: 7.3/ 16.9 / 1.1%; B: 5.5 / 5.9 / 6.5%; B-cells in THY highly co-expressed CD5. Discussion: RTX produces different effects in the target tissues of this organ-specific autoimmune disease. A single RTX course could obtain B depletion in the orbit and amelioration of TAO but not B depletion in thyroid, where we found. CD19+CD5+ cells.

## IMMUNOPHENOTYPING OF REGULATORY T CELLS

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Identification of $CD4^+/CD25^{+\ bright}$ cells has helped define function and immunophenotype of regulatory T lymphocytes (Tregs). This study compared immunophenotypes of selected $CD4^+/CD25^{+bright}$ and $CD4^+/CD25^{+low}$ cells. Materials and Methods: After giving informed consent 7 donors provided haematopoietic stem cells for allogeneic transplant. Mononuclear cells (MNC) were collected from lymphocytapheresis products using a continuous flow cell separator. $CD^+/CD25^{+\ bright}$ cells were selected by immunomagnetic separation (Automax system, Miltenyi Biotec, Bologna) and compared with non-reactive $CD4^+/CD25^{-/low}$ cells. These monoclonal antibodies were used: CD25 ((Miltenyi Biotec), FoxP3 (Biolegend), CD133, CCR7, CCR5 (BD Biosciences), CD52 (CALTAG Laboratories), CD1a, CD3, CD2, CD5, CD7, CD4, CD10, CD15, CD127, CD90, CD95, CD117, CD135, CD62L, CD45RA, CD45RO, ZAP70 (I.L., Milan). Immunophenotypes were assessed by flow cytometry analysis (FC500, I.L., Milan). Results: No significant differences emerged in CD2, CD7, CD5 antigen expression. CD1a, CD10, CD15, CD90, CD117, CD135, CD133 antigens were not expressed. Conclusions: Immunophenotyping showed that CD4/$CD25^{bright}$/FoxP3/CD127 antigen expression characterises the Treg phenotype. Molecular analysis and functional studies demonstrated almost total inhibition in mixed lymphocyte cultures. Almost all Tregs expressed CD52. CD52 expression has major implications in therapy for some malignant lymph node diseases and in conditioning regimens to allogeneic bone marrow transplantation.

| | $CD4^+/CD25^{+bright}$ | $CD4^+/CD25^{+low}$ |
|---|---|---|
| CD45RA | 1.9% ± 0.6 | 5.2% ± 3.9 |
| CD45RO | 98.2% ± 1.2 | 95.4% ± 2.7 |
| CD52 | 93.7% ± 3.5 | 96.5% ± 6.1 |
| CD62L | 91% ± 1.1 | 81% ± 2.1 |
| CD95 | 8.5% ± 8 | 4.3% ± 1.9 |
| CD127 | 0% ± 0.1 | 8.9% ± 5.7 |
| CCR5 | 42.3% ± 14 | 12.1% ± 8.1 |
| CCR7 | 2.2% ± 1.9 | 2.1% ± 1.8 |
| FoxP3 | 80% ± 6 | 59% ± 6.8 |
| ZAP70 | 96% ± 9 | 95% ± 8 |

## CD124 (IL4Rα) ON B CELLS: CHANGES IN IDIOPATHIC MEMBRANOUS NEPHROPATHY (IMN) UNDER RITUXIMAB TREATMENT

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Deposition of IgG4 immune complex in the glomerular capillary walls is the determinant factor of IMN pathogenesis, the main cause of adult nephrotic syndrome. Recent studies have defined the IMN as a Th2 immune disorder and IL4 has a key role in Th2 reaction and in IgG4 subclass switching. Furthermore, a glomerular interstitial B cell (CD20+) infiltration has been detected in the disease, so a rituximab treatment has been employed for the specific depletion of B cells. Our analysis by PCR-SSP and comparison between patients (n = 44) and control group (n = 124) has show that allele T -590 IL4, is 45% vs 7% (OR = 10,5 $p < 10^{-6}$), thus exist a grate link between IL4 promoter genetic variation end predisposition to IMN. So, on peripheral blood, of patients with persistent (>6 mo) urinary protein excretion (>3,5 g/24h) under treatment with rituximab (1 infusion of 375 $mg/m^2$), we analysed by flow-cytometry lymphocyte population, CD20 level and state of CD124 for therapy monitoring and observed the expression level of CD19 CD20 and CD124 at 0-3-6-12 mo or plus of the therapy start.

Data show the existence of three B cell groups, $CD20^{dim}$ CD124-, $CD20^{bright}$ CD124- $CD20^{bright}$ $CD124^{dim}$. In latter group we observed a correlation between the CD20/CD124 level expression (n = 29 r = 0,52 p = 0,001) in basal patients and in patients at 1 yr or plus from rituximab treatment. In patients with CD4/CD8 ratio >2, as compared to patients with a normal ratio (n = 22 2,9+/−0,8 vs n = 20 1,5+/−0,4 p < 0,001 t = 6,6) we observed that G −1082 IL10 (high producer), was 66% vs 35% (OR = 3,01 $p = 1{,}7 \cdot 10^{-3}$), thereby showing its involvement in the immunological activity of B cells (Ig production).

Data confirm Th2 hypothesis and suggest the existence of IL4 effects, via CD124, on CD20 expression and of a link between IL4 and B cell response. We conclude that IL4R has a central role in the insurgence of the disease and may have a future role for new terapeutical strategy.

## PHENOTYPICAL AND FUNCTIONAL CHARACTERIZATION OF PERIPHERAL NATURAL KILLER (NK) CELLS IN PULMONARY TB PATIENTS

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M. tuberculosis is a pathogen that infect up to one-third of world's population and 10% of infected individual

will develop active disease. NK cells have an important role in innate immune response against intracellular pathogens and it has been described that compromised control of microorganism spreading are associated, in some instances, to alterations of NK cell function. In order to analyze if ex-vivo peripheral NK cells from *M. tuberculosis* infected patients are associated with phenotypical and/or functional perturbations of NK subsets, we study a cohort of diagnosed pulmonary TB patients in comparison with a control group represented by healthy uninfected donors. Phenotypical analysis of NK cells was performed on PBMC by four-colour cytofluorometry using monoclonal antibodies specific for activating and inhibithory NK receptors. In addition intracellular γIFN production was evaluated after stimulation with NK-sensitive tumor target cells and/or with anti-Natural Cytotoxicity Receptors (NCR) mAbs. Preliminary data show that the expression of few surface receptors on NK cells are significantly different in TB patients compared with healthy donors. If these phenotypical alterations could also affect NK functional capacity is actually under investigations.

## DIAGNOSES OF ADVERSE DRUG REACTIONS BY FLOW CYTOMETRY: BASOPHILS DEGRANULATION TEST vs BASOPHILS ACTIVATION TEST

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The Adverse Drug Reactions (ADR) are classified into three main classes: dose-dependent, bizarre and complex reactions. The first class includes toxic effects in consequence of overdoses, collateral effects and drugs interaction. Second class of ADR is unpredictable, dose-independent, and it is evidenced as allergic reaction IgE-mediated (very rarely) and not IgE-mediated (most frequently), or as not allergic hypersensitivity. Third class of ADR is observed only after a persistent induction and the mechanisms are unknown. The study of basophils reaction by flow cytometry helps us to evidence a good part of the ADR both IgE- and not IgE-mediated. The procedure is good standardized and not involve in risk for subjects. In this work we introduce the results of a study on basophils degranulation and activation process, performed in parallel on a large number of patients who report ADR to antibiotics. Degranulation process on basophils is evidenced through the MoAb CD63 that mark lisosomial protein gp53 expressed on basophils membrane surface after the granule release. Basophils activation is evidenced through the ENPP-3 (CD203c) expression, known as a marker of specific basophils activation in allergic reaction. Our results evidenced a difference in preferential mechanisms induced form different drugs in sensitive subjects, and suggest different means for CD63, and CD203c expression on basophils as a result of drugs stimulation; besides it can be supposed a relationship of degranulation (CD63) and activation (CD203c) process respectively with early and late reaction.

## PHOSPHATIDYLCHOLINE-SPECIFIC PHOSPHOLIPASE C EXPRESSION IN HUMAN NATURAL KILLER CELLS

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We recently reported evidence of phosphatidylcholine-specific phospholipase C (PC-PLC) involvement in NK cell-mediated cytotoxicity and in lytic granule exocytosis. In the present study, different subpopulations of human PBLs were investigated in relation to PC-PLC enzyme expression. In this context, we identified two different NK cells subpopulations, $CD56^{dim}$ $PC\text{-}PLC^{bright}$ and $CD56^{bright}$ $PC\text{-}PLC^{low/-}$ cells, corresponding to distinct subsets with cytolytic and immunoregulatory functions, respectively. Interestingly, the PC-PLC expression level on the NK membrane surface correlated closely with that of the CD16 receptor (FcgammaR-IIIA), suggesting a possible relationship between enzyme membrane expression and NK cell maturation. Analysis of PC-PLC and CD16 distribution in NK cell plasma membrane demonstrated that the proteins were physically associated and partially accumulated in lipid rafts. CD16 cross-linking resulted in an increase of PC-PLC enzymatic activity within 5–10 minutes after stimulation. Pre-incubation of NK cells with a PC-PLC inhibitor, D609, determined a specific decrease both in CD16 receptor and PC-PLC enzyme expression on the plasma membrane. CD16-mediated cytotoxicity was also reduced after D609 incubation. Taken together, these data suggested that the PC-PLC enzyme could play an important role in regulating CD16 membrane expression. PC-PLC seemed to be therefore involved in the CD16-mediated cytotoxicity and in the early steps of CD16 signal transduction.

## CD203c UPREGULATION IN THE DIAGNOSIS OF ALLERGY TO QUINOLONES

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Quinolones are responsible for adverse reactions both of immediate and delayed type, but in vivo and in vitro tests for quinolones are not yet standardized. The aim of our study was to demonstrate a specific IgE-mediated immune response in patients undergoing allergy testing for adverse reactions during quinolones therapy. To such extent, we used the "allergenicity kit" based on the upregulation of CD203c (ecto-nucleotidepyrophosphatese/phosphodiesterase 3, marker of basophils and mastocytes), induced by quinolones. We studied 13 consecutive patients whose clinical history was suggestive of adverse reactions to quinolones (levoxacin, pipemic acid, cinoxacin, moxifloxacin, nitrofurantoin). In all the tests a negative control, selected on the basis of personal history among the drugs tolerated by the patient, was used. In 8

cases, in which the medical history was strongly suggestive of severe drug reaction, the test was positive. In the other cases, in which the history was not probative or uncertain, the test was negative. These data suggest that the "allergenicity kit" might be useful for the identification of IgE-mediated severe reactions to quinolones and potentially to other drugs.

## A SIX-COLOR POLYCHROMATIC FLOW CYTOMETRY TO EVALUATE HUMAN BASOPHIL ACTIVATION

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An in vitro basophil activation assay model by using a two-laser 6-color polychromatic flow cytometry (PFC) is described. $K_2$-EDTA anticoagulated peripheral blood from 80 screened donors was used and cells were obtained by collecting buffy coat layers washed out from plasma without either separation polymers or immuno-purification. In each of 22 tests 1500–2500 basophils were sorted as $CD45APCCy7^{dimly}$ leukocytes in a $CD123PECy5^{bright}$/$HLADRPECy7^{neg}$ immunological gating by using a FACScanto flow cytometer. Any spontaneous activation was prevented by using apyrogenic reagents at 4°C and sterile disposable plasticware. Resting homogenous populations responsive to agonist challenge were obtained in all the assays performed. Biparametric plots, mean fluorescence (MFI) and % $CD63^{bright}$ were used for time-course and dose response curves. With 100 nM fMLP, CD63FITC up-regulation occurred very early, exhibited very high MFI but involved only 30% basophils while CD203cPE upregulated few minutes later and at a 10× lower agonist dose. With $2\times10^{-6}$ M anti-human IgE only 14% were $CD63^{bright}$ while all basophils up-regulated CD203c, thus evidencing a differential activation pattern Agonists changed slightly CD13APC or CD69APC activation pattern while they did not affect gating markers except for CD45. This PFC protocol, allowing an overall and systemic approach on basophil activation within the same experimental condition, is an affordable tool to investigate the physiopathology and pharmacology of basophil activation.

## FLOW-CYTOMETRIC CHARACTERIZATION OF PERIPHERAL BLOOD DENDRITIC CELLS IN COELIAC DISEASE

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Background and aim: Peripheral blood DCs do not represent a homogeneous cell population, but a mixture of at least two subsets, the $CD11c^{bright}$/$CD123^{dim}$ subset that represents the myeloid-derived DCs (DC1) responsible for a Th1 response, and the $CD11c^{-}$/$CD123^{bright}$, designed as lymphoid-derived DCs (DC2), having potent Th2 stimulatory function. Recently, a panel of monoclonal antibodies direct towards four novel human DC antigens, called BDCA, was generated, BDCA-1 and -3 being specific for myeloid type, and -2 and -4 specific for lymphoid type. In CD the cytokine pattern and the strong association with the class II HLA molecules emphasise a Th1 polarization. Up to now, no information about the subsets of circulating DCs in CD is available, therefore, we aimed to carry out a phenotypical characterization of this cell population in this pathological condition. Patients and Methods: Peripheral blood samples from 13 untreated and 15 treated CD patients and from 18 healthy controls were used. DC subsets were identified at FACS analysis (EPICS-XL, Coulter) by using two approaches: a) calculating the % of circulating DCs when gated as lin-, HLA-DR+ and identified as DC1 and DC2 according to expression of CD11c and CD123, respectively; b) by using three specific markers BDCA-1 (CD11c-high/CD123-low myeloid DCs); BDCA-2 (CD11 c-neg/CD123-high lymphoid DCs) and BDCA-3 (CD11c-low/CD123 neg myeloid DCs) (Blood Dendritic Cell Enumeration Kit, Miltenyi). Statistical analysis was performed by the *t* test. Results: In both untreated and treated CD patients a significant reduction of the entire DC population in comparison to healthy controls was observed either as percentage or as absolute value ($p=0.01$ and 0.009 in active CD *vs* controls, $p=0.04$ and 0.002 in treated CD *vs* controls). In addition, a significant increase of the DC1 subset ($p=0.01$ for both values in active CD *vs* controls, $p=0.011$ for both values in treated CD *vs* controls) with a parallel reduction of the DC2 subset ($p=0.01$ and 0.001 in active CD *vs* controls, $p=0.022$ and 0.001 in treated CD *vs* controls) with a parallel increase of the DC1/DC2 ratio were found. Conclusion: The presence of myeloid polarised DCs in both active and treated CD, accompained by a concomitant reduction of the total amount of circulating DCs, strengthens the role of this subset of cells in polarizing the immune response towards a Th1 profile and suggest that the loss of tolerance in this condition may be a primitive defect.

## USE OF MULTI-DIMENSIONAL FLOW-CYTOMETRY TO CHARACTERIZE CIRCULATING IMMUNE CELLS IN CRONH'S DISEASE PATIENTS FOLLOWING INFLIXIMAB THERAPY

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Background and aim: Crohn's disease (CD) is a recurrent and disabling chronic inflammatory bowel disease that affects a growing number of subjects in western countries. Recent evidence has shown that determination of the intracellular cytokine pattern of T lymphocyte and the immune phenotype of dendritic cells in peripheral blood of CD patients is indicative of disease activity. Furthermore, it is well known that Infliximab, the anti-tumor necrosis factor (TNF)-α monoclonal antibody, is highly effective in steroid-dependent and -refractory patients as well as in fistulizing disease, but the mechanisms underlying its effects are not fully understood. Thus, we investigated the modifications of circulating T lymphocytes and dendritic cells induced by

Infliximab treatment. Patients and Methods: Peripheral blood samples were obtained from eight patients (5 males) before and at 12$^{th}$ week of therapy (I-CD), fourteen patients (8 males) following traditional therapy, i.e. mesalazine, steroids, antibiotics (T-CD), and nine healthy volunteers (3 males). Quantification and characterization of T cells and dendritic cells was carried out by multidimensional flow-cytometric analysis (EPICS-XL, Coulter) together with the measurement of TNF-α, interferon (IFN)-γ, interleukin (IL)-4 and IL-10 producing T cells. Results: In I-CD patients before Infliximab, the count of white blood cells, CD8$^+$, TNF-α and IFN-γ producing T cells turned out to be significantly higher in comparison to T-CD and healthy subjects, whilst that of IL-4$^+$ cells resulted lower. No modification was observed for IL-10 expressing cells. Interestingly, a significant depletion of the entire dendritic cell population, mainly the plasmacytoid subset, in all CD patients in comparison to controls was evident. All these parameters, with the exclusion of the number of plasmacytoid dendritic cells, reached normal values after treatment. Conclusion: Our results suggest an important immunosuppressive and immunomodulatory action of Infliximab on peripheral blood T cells and dendritic cells, and indicate that characterization of circulating immune cells is a useful additional diagnostic tool in the management of CD patients.

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ANALYSIS OF γδ T CELLS RESPONSE TO BRAIN TUMORS: IMPLICATIONS FOR THERAPY

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γδ T cells present some particular characteristics and link innate and acquired immunity.

In humans 90% of circulating γδ T cells express the Vγ9Vδ2 TCR rearrangement and recognize non peptidic antigens in a MHC-unrestricted manner. After antigen recognition, activated Vδ2 T cells rapidly proliferate, produce high levels of cytokines and chemokines and can differentiate in cytotoxic effector cells. Several studies show that Vδ2 T cells are able to kill tumor cells, such as lymphoma, colon- lung- and renal carcinomas and glioblastoma.

In this study we have analyzed in healty donors γδ T cells response versus five brain tumor cell lines, two astrocytomas (T67, T70) and three glioblastomas (U87, U373, U251).

We have observed that Vδ2 T cells and Vδ2 T cells lines can recognized U251 tumor line releasing pro-inflammatory cytokines (IFN-γ and TNF-α). The treatment of all brain tumors cell lines with drug Zoledronate is able to activate circulating Vδ2 T cell and Vδ2 T cell lines to release IFN-γ and TNF-α. This response seems to be blocked by Mevastatin confirmed the involvement of mevalonate cycle in the mechanism of recognition between γδ T cells and tumors.

These results show the possibility to induce a strong response of Vδ2 T cells in term of release of cytokines in the treatment of tumor.

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ANALYSIS OF THE INTERACTION OF MATURING NK CELLS WITH REPLICATING VIRUSES: STRONG UPREGULATION OF NCR EXPRESSION AND OF CYTOTOXICITY BY HSV-1, NOT BY HIV-1

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Compromised control of microorganism or tumor spreading are associated in some instances to alterations of NK cell function. Mechanisms underlying this dysfunction are still unclear and may involve perturbations of NK cell differentiation in the presence of pathogen replication. We studied an "in vitro" model of NK cell differentiation from highly immature CD34+Lin- precursors in the presence of replicating HIV-1 and HSV-1. No significant downregulation in NCR molecule expression and no impairment in the cytolytic capability of NK precursors were evident with HIV-1. On the contrary, the presence of productively HSV-1-infected cells induced an increased surface NCR molecule density resulting in significantly increased cytolytic activity. Importantly the HSV-mediated effect, is limited to maturing NK cells, and does not involve peripheral blood mature NK. Analysis of TLR expression on NK cells revealed augmented expression of the intracellular TLR9 molecule in CD56+CD33− NK cell precursors in the presence of replicating HSV-1, but not of HIV-1. Thus, virus replication at sites of NK cell precursor development (e.g.: lymph node, bone marrow) may have different outcomes depending on the interaction between invading viruses and maturing NK cells and may contribute to success or failure to control virus replication/spread.

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THYMOPOIESIS AND TCRVβ DEVELOPMENT IN THYMOMA WITH AND WITHOUT MYASTHENIA GRAVIS (MG) AND MODULATORY EFFECTS OF STEROID THERAPY

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MG is an autoimmune disease mediated by autoantibodies that interfere with the function of the neuromuscular junction. MG is often associated to thymoma, a tumor of thymic epithelial cells harboring non-neoplastic lymphocytes. We show that certain alterations of thymopoiesis, i.e., an excess of immature CD4$^+$CD8$^-$CD3$^-$ thymocytes and an abnormal TCRVβ development through CD4$^+$CD8$^+$ to CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ transition occur likewise in MG- and non MG-associated thymomas, thereby excluding their relevance in MG onset. Terminal thymopoiesis, i.e., CD4$^+$CD8$^-$ /CD8$^+$CD4$^-$CD3$^+$CD45RA$^+$ thymocyte production, tends to be skewed toward CD4$^+$ compartment in MG-associated and CD8$^+$ compartment in non MG-associated thymoma. Importantly, thymic output is not increased

in MG-associated thymoma indicating that thymoma-produced $CD4^+$ lymphocytes are dispensable in disease maintenance, at variance with previous conclusions. In contrast, thymic export of naïve $CD8^+$ lymphocytes is significantly increased in non MG-thymoma. These cells probably represent thymoma-derived cytotoxic lymphocytes thought to be involved in an early stage of autosensitization before overt MG. In the second part of our study, we show a) that steroid administration to MG patients favors terminal thymopoiesis and b) steroid resistance of thymocytes of certain MG patients, known for decades, depends on microenvironmental clues.

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CD8+CD28− T REGULATORY LYMPHOCYTES INHIBITING T CELL PROLIFERATIVE AND CYTOTOXIC FUNCTIONS INFILTRATE HUMAN CANCERS

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Among mechanisms responsible for tumour immune escape great relevance is attributed to tumour infiltration by CD4+CD25+ T regulatory cells, infact these cells are present in tumour infiltrating lymphocytes (TILs), inhibit anti-tumour immune responses and their rate of infiltration correlates with tumour progression. Recently, another regulatory T lymphocyte subpopulation constituted by CD8+CD28− T lymphocytes (Ts) has been identified in humans, but scanty information exist concerning their involvement in cancer. Our study is aimed to study CD8+CD28− T cells in the peripheral blood and in primitive or metastatic lesions of cancer patients in comparison with CD4+CD25+ T lymphocytes. We observed that TILs purified from 40 tumor specimens are mainly constituted by CD8+CD28− Ts lymphocytes able to inhibit both T cell proliferation and cytotoxicity, whereas the concentration of CD4+CD25+ T reg lymphocytes in primitive tumour lesions was lower than that of CD8+CD28− T suppressor cells. The infiltration of Treg cells was directly dependent on tumour cell function being present in metastatic but not in metastasis free satellite lymph nodes. The tumour infiltration by regulatory T cells could be induced by: in situ generation (via cytokine production) and recruitment from the periphery (via chemokine secretion). These results have pathogenic relevance and implication for immunotherapy of cancer.

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TNF FAMILY MEMBER EXPRESSION DURING $CD56^{BRIGHT}$ NK CELL DIFFERENTIATION IN VITRO

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It is known that IL-15 is able to induce the in vitro development of the $CD56^{bright}$ NK cell subset from $CD34^+$ hematopoietic progenitors (1), and also the activation of NK cells (2). To this regard, Tumor Necrosis Factor (TNF) family (receptor and ligand) members have been described to be up-regulated on mature NK cells upon cytokine stimulation (2), and their appearance would follow a specific sequence of expression (1, 2). In the present report, we have investigated the expression of TNF ligand and receptor members on $CD56^{bright}$ NK cells generated in vitro from $CD34^+$ PB hematopoietic progenitors. After 25-30 days of culture with Flt3L and IL-15, $CD56^{bright}$ NK cells generated in vitro expressed TRAIL (at low density), Fas (CD95), TNF-R2, TNF-R1 (at low density) and TRAIL-R4 on a small subset, while FasL, TRAIL-R1, -R2, and -R3 were undetectable. In order to monitor the state of activation of differentiating $CD56^{bright}$ cells, expression of TNF family molecules was determinated after further 15 days of secondary cultures with IL-15 administration. Resembling activated peripheral blood $CD56^+$ NK cells, $CD56^{bright}$ NK cells maintained Fas, TNF-R1 and TNF-R2 molecule expression and up-regulated FasL and TRAIL-R2, becoming sensitive to TRAIL induced apoptosis. Our findings define a specific sequence of expression of TNF family members in in vitro generated $CD56^{bright}$ NK cells that is similar to that induced in mature activated $CD56^{bright}$ NK cells. In particular, we found that surface TRAIL molecule is induced early during in vitro NK cell differentiation/activation, while FasL and TRAIL-R2 are later marker of activation.

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FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) AS A USEFUL TOOL IN STUDYING CD95/FAS-EZRIN ASSOCIATION DURING LYMPHOCYTE ACTIVATION

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RATIONALE: Flow cytometry is a very sensitive method to detect fluorescence resonance energy transfer (FRET) that allow us to follow cell-by-cell molecular associations in intact cells. METHODS: Freshly isolated human lymphocytes were stained with anti-CD95/Fas and anti-ezrin antibodies conjugated to phycoerythrin (PE) or indirectly labeled with Cy5. The FACSCalibur (BD) equipped with a 488-argonion laser and a 635-diode was used for sample analysis. RESULTS: The polarization of Fas receptors, through an ezrin-mediated association with the actin cytoskeleton is a key intracellular mechanism rendering activated T lymphocytes susceptible to Fas-mediated apoptosis. We studied how IL-2 treatment modulates organization of Fas molecules in the cell membrane of lymphocytes. To this purpose, we daily analyzed human lymphocytes cultured with or without IL-2 for 6 days. We found that, upon IL-2 treatment, cells showed a time dependent CD95/Fas polarization and ezrin-CD95/Fas co-localization

(by confocal microscopy analysis). In addition, a time-dependent increase of FRET efficiency after IL-2 treatment between fluorochromes used for labeling CD95/Fas and ezrin demonstrated that IL-2 induced a progressive association of these molecules (also confirmed by co-immunoprecipitation method). These data showed that after IL-2 treatment i) CD95/Fas receptors are expressed in a polarized manner on the cell surface and that ii) a direct interaction between CD95/Fas molecule and ezrin occurred. This association seems to represent a prerequisite for apoptotic signal transduction. CONCLUSION: The FRET assay allows the direct investigation of the dynamic association of CD95/Fas receptor with ezrin molecule during IL-2-induced lymphocyte activation.

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EVALUATION OF LYMPHOCYTES POPULATION IN GASTRIC BIOPSIES: AN IMPROVEMENT BY A CYTOFLUORIMETRIC APPROACH

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Background: It is well know that gastritis has a broad histopathological and topographical spectrum and leads to patterns of disease. The alterations in normal stomach mucosa due to an inflammatory process are usually characterized by an evaluation in light microscopy. For this reason, the count of the lymphocytes number in these specimens is often subjective and not completely reliable. There are no data on a cytofluorimetric approach in this field and our aim is to purpose an objective quantification of the lymphocytes count by flow cytometry. Methods: Biopsies (diameter of 2–4 mm, depth of 2–3 mm) were collected from gatric antrum (#33), corpus (#15) and fundo (#2) of 50 patients (average age 60 years old), who underwent endoscopy for dyspepsia, were included in the study. The Histopathological evaluation was performed by Hematoxylin & Eosin (H&E). Flow cytometry (FC) by EpicsXL (Coulter) and Immunoistochemistry (IHC) analysis for CD45, CD3, CD19, CD4, and CD8, were subsequently performed. Results: The histopathological evaluation by H&E revealed the follow diagnosis: 72% (36/50 Quiescent Chronic Gastritis, 22% (11/50) Active Chronic Gastritis, 6% (3/50) normal mucosa. Considering all the patients, we found Helicobacter Pylori in 7/50 (14%) cases. On these cases we performed FC counting 10000 cells for sample while in IHC we considered 5 fields for slide at ×400 magnification. We found a general correlation between FC and IHC results considering all the markers analysed for lymphocytes (correlation coefficient 0.97). Conclusion: Data obtained show that an accurate evaluation by flow cytometry of the lymphocytes number could give an objective and fast quantification of the lymphocytes count. This finding could be useful in the evaluation of inflammatory conditions which leads to gastritis and reflect different clinical behaviours.

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INCREASED FREQUENCY AND SUPPRESSIVE FUNCTION OF T REGULATORY CELLS IN MULTIPLE MYELOMA.

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Multiple Myeloma (MM) and Monoclonal Gammopathy of Undetermined Significance (MGUS) are B-cell immunoproliferative disorders characterized by the expansion of plasma cells and late B cells in the bone marrow, associated with an impaired T-cell immune response. Since T regulatory cells (T regs) play an important role in the modulation of T cell response, we evaluated their potential contribute in MM. Expression of FoxP3, a transcriptional factor required for regulatory and suppressive function, was used to assess by flow cytometry the proportion of CD4 and CD8 T regs in the peripheral blood of normal donors and patients with MGUS and MM at different clinical stage.

An increased frequency of $CD4^+$- and $CD8^+$-$FoxP3^+$ T regs was observed in patients with active MM compared to patients with stable MM, MGUS and normal donors. In addition, analysis of surface CTLA-4 (cytotoxic T lymphocyte-associated protein-4) expression on $FoxP3^+$ cells demonstrated a higher proportion of double-positive cells in patients with active MM, implying that T regs are functionally active. Data were further confirmed by real-time PCR experiments. Finally, functional in vitro studies demonstrated the ability of myeloma T regs to suppress T-cell proliferation. Overall results suggest a potential role of T regs in suppressing T-cell immune response in MM.

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AN ALTERNATIVE METHOD TO EVALUATE THE STRESS IN PIGS USING A CYTOFLOUROMETRIC ANALYSIS

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The stress condition in pigs is studied estimating the natural immunity of the animal (semi-quantitative titration of the complement, titration of the serum lysozime, bactericidal activity of the serum), but nowadays, in parallel with it, we have tried to study the stress of these subjects estimating the percentual increase, at hematic level, of cells CD25+ inside of the population of lymphocytes T CD4+.

The receptor CD25 is the receptor ·-chain for IL-2 and its over expression inside of population of lymphocytes T is likely to happen when these last ones are subordinated to stress conditions, such as, for example, the stimulation with LPS.

The estimation of the percentage of lymphocytes CD4+ and CD25+ has been made through cytoflourometric methods using monoclonal antibodies marked by two fluorochromes (FITC and PE).

This study compares the percentages of cells CD4+ and CD25+ obtained from two different groups of animals: the first one is represented by animals bred in good sanitary conditions and in an habitat in accordance with spe-

cies requirements, the second one is composed by animals which come from a factory farming characterized by uncertain sanitary conditions.

The data gathered by means of cytoflourometric analysis are compared with those deriving from the semiquantitative titration of the complement, from the titration of the serum lysozime and from the bactericidal activity of the serum; the aim of this comparison is to point out any correlation between the data gathered by means of cytoflourometric analysis and the stress condition of the animal.

## SPECIFIC T-CELL THERAPY FOR VERY HIGH RISK OSTEOSARCOMA PATIENTS

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Background: The prognosis of relapsed osteosarcoma patients remains poor despite recent advances. The expression of MAGE, SSX and SART-3 family antigens in osteosarcoma cell lines and in fresh osteosarcoma tissues, allowed the development of immunotherapeutic strategies. We studied the feasibility of creating osteosarcoma specific cytotoxic T lymphocytes (CTLs) from healthy donors as preliminary conditions for their use in adoptive immunotherapy for high-grade osteosarcoma patients. Methods: We used HLA-identical osteosarcoma cell lines or autologous dendritic cells (DCs) maturated by using the classical cytokines cocktail or Zoledronic acid (ZA) 1 μM. Both types of mature DCs were pulsed with irradiated (70 Gy) tumor cells to stimulate PBMCs. IL-2, IL-7, IL-12 and IL-15 were added. After three stimulations we evaluated the phenotype, the specificity and the cytokine secretion of the expanded CTLs. Results: We succeeded in developing anti-osteosarcoma specific CTLs, which are CD8+ and secrete IFN-γ only against the osteosarcoma line used for the stimulation. In particular, CTLs generated with HLA-identical osteosarcoma cell lines recognized the line used and also 4/5 of the other osteosarcoma cell lines tested and are stopped by HLA-class I blocking antibodies. Moreover, in CTLs generated with autologous ZA-maturated DCs the majority of effector cells (>70%) were CD3+/CD8+ (Fig. 1). Conclusions: These results demonstrate that our experimental approach is suitable for efficiently generating and expanding anti-osteosarcoma specific CTLs to be used for adoptive immunotherapy.

## SEEKING A NEW ROLE FOR AN OLD MOLECULE: PARP-1 AND THE GENERATION OF REGULATORY T CELLS

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$CD4^+CD25^+$ regulatory T cells (Treg) contribute to the maintenance of immunologic self-tolerance by inhibiting the activation of auto-antigen reactive T cells. Treg cell development occurs in the thymus, is dependent on Foxp3 expression and sustained by high affinity TCR-MHCII peptide complex interactions. Some studies suggested a possible role for poly(ADP-ribose)polymerase-1 (PARP-1), an enzyme that catalyzes the attachment of ADP-ribose units to target proteins, in the regulation of immune functions. In our study, we investigated phenotype and functional characteristics of different lymphocyte subpopulations in PARP-1KO mice. We observed that lack of functional PARP-1 leads to an expansion of the Treg cell population in the spleen, as revealed by CD25 and Foxp3 expression analysis. As a result, spleen cells from PARP-1KO mice produce less IL-2 and display a reduced cell proliferation rate in response to TCR/CD3 stimulation. Interestingly, on a per cell basis Treg cells from PARP-1KO mice show no differences in the inhibitory function as compared to wild type Treg cells. In PARP-1KO thymus, the $CD4^+CD8^-$ sub-population also contains a higher number of $CD25^+Foxp3^+$ Treg cells as compared to wild type littermates thymuses. Alterations in the frequency of other subpopulations were not evident. Altogether, these findings suggest that lack of PARP-1KO may favour the thymic development of Treg cells. We challenged this hypothesis in a competitive bone marrow (BM) chimera assay. $RAG2\gamma_c$ double KO mice were transplanted with a mixture of BM cells from PARP-1KO(Thy1.2) and wild-type C57Bl/6(Thy1.1) mice. Results show that twelve weeks after BM transplantation the majority of the thymic $CD4^+CD8^-Foxp3^+$ Treg cells were generated from PARP-1KO BM cells. In conclusions, our results are the first evidence that lack of PARP-1 favours the development of Treg cells and thus it might be a target molecule for new strategies in the control of tolerance-breaking pathologies.

## A MULTIPARAMETRIC ANALYSIS TO STUDY THE LYMPHOCYTE SUBSETS BY USING A COMPARATIVE METHOD

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The immune system is a multicomponent dynamic entity, very sensitive to many exogenous and endogenous stimuli. Lymphocytes are the pivot cells of the system and are associated with the specificity of the immune response; they differentiate in many subsets expressing different sophisticated functions. Their amount and activities change sensitively with the age.

Due to complexity and diversity of these components it is crucial the employment of multiparametric investigation technologies in order to acquire a systemic vision of the global immune activity both physiological and pathological.

We routinely determined lymphocytes subsets by using flow cytometry (FACScanto, Becton Dickinson) and monoclonal antibodies (BD Bioscience) of six different fluorochromes. The usual panel employed is: CD45, CD3, CD4, CD8, CD16, CD56, CD57, CD19, CD23, CD27, CD5, HLA-DR.

In order to establish standard references for the various subset profiles, we created a data bank composed by data from the afferent subjects to our laboratory (about 4500). To evaluate the parameters significance we compare values of the single subject with a group of subjects (about 200) of the same age. The obtained series of values (from 0 to 100), corresponding to the percentile of all parameters, are plotted in a graphic representation, as a fingerprint, which allows an immediate evaluation of the totality of parameters and their shifts from the reference group. To follow up the subjects we plotted in the same graphic the first and the second analysis.

## TARGETING REGULATORY T CELLS (TREG) IN VITRO BY HUMANIZED AND CHIMERIC ANTI-CD25 MONOCLONAL ANTIBODIES

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Suppression by $CD4^+CD25^+Foxp3^+$ Treg requires IL-2 from responding T cells but high dose exogenous IL-2, similar to that administered to cancer patients, can abrogate suppression and hamper effector T cell proliferation by favouring the concomitant expansion of Treg. Anti-CD25 monoclonal antibodies (MoAbs) deplete Treg and facilitate anti-tumour immune response in mice. We tested whether chimeric (basiliximab, Simulect™) or humanized (daclizumab, Zenapax™) anti-CD25 MoAbs currently used to target $CD25^+$ effector T cells in solid organ transplantation would prevent the suppressive function of Treg. Disappointedly, both anti-CD25 MoAbs dose-dependently decreased polyclonal T cell proliferation, indicating they were preferentially targeting de novo CD25 expressing activated effector T cells rather than Treg. Effector T cells but not Treg express IL-15 and IL-7 receptors. Despite this, addition of exogenous IL-15 alone or in combination with IL-7 to anti-CD25 MoAb treated cultures, did not reduce Treg, as assessed by Foxp3 expression (flow cytometry and quantitative RT-PCR) although it did restore proliferative response. We are now culturing Treg depleted T cells to test whether anti-CD25 MoAbs in the presence of IL-15/IL-7 may prevent de novo Treg development.

## DETECTION OF CIRCULATING MYELOID (mDC) AND PLASMOCYTOID (pDC) DENDRITIC CELLS (DCs) SUBSETS BY FLOW CYTOMETRIC (FCM) ANALYSIS IN SEPSIS

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Introduction: The role of DCs in sepsis is poorly understood. Our aim was to investigate the dynamic changes of peripheral blood DC (PBDC) and of their mDC and pDC subsets at the onset and during the evolution of severe sepsis. Methods: We included 27 severe septic pts, 10 surgical pts (elective aortic aneurysm surgery) and 20 healthy controls (HC). In sepsis samples were collected at diagnosis and at clinical resolution or before death; in surgical pts, on the 1th post-op.day. By FCM analysis DC are recognized by immunoglobulin-like-transcript-3 (ILT3) and differentiated in mDC and pDC subsets by CD11c and CD123. Results are median and IQR of absolute DC numbers ($\times 10^6$/L). Results: At diagnosis, in sepsis mDC decreased ($p<0.001$ vs HC) while pDC increased ($p=0.03$),with a reduced mDC/pDC ratio ($p<0.001$). On the contrary, after surgery both mDCs and pDCs diminished ($p<0.001$), without mDC/pDC ratio change. At diagnosis, no difference in mDC was found between 16 septic survivors (SS) and 11 non survivors (SNS), while pDCs were significantly higher in SNS ($p=0,03$). Moreover, with respect to HC, pDC were significantly higher only in SNS ($p=0.002$). In the final sample mDC were unchanged in SNS, but selectively increased in SS ($p=0,001$); no differences pDC between the two samples were observed both in SS and in SNS. As a result, the ratio of mDCs to pDCs was unchanged in SNS whereas it significantly increased in survivors ($p<0,001$). Conclusion: Our findings suggest that PBDC are affected by severe sepsis. Moreover the two subsets are differentially impaired. These alterations are sepsis specific and survival seems linked to recostitution of the normal mDC/pDC ratio.

## DETECTION OF CIRCULATING DENDRITIC CELLS SUBSETS BY FLOW CYTOMETRY IN PERIPHERAL BLOOD OF HEALTHY SUBJECTS: TWO DIFFERENT APPROACHES

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Background: Dendritic cells (DCs) are antigen presenting cells that play a crucial role in antitumor immune response. DCs can be detected in peripheral blood (PB) and divided into 2 subsets identified according to their function: myeloid DCs (DC1) and plasmocytoid DCs (DC2). As preliminary phase of a clinical study on cancer patients, we wanted to define DC and DC subset values in healthy subjects (HS). Methods: Samples of PB from 50 HS (M/F: 25/25, median age 40 yrs) were analysed. The mean values of the percentage and of the absolute number of DC subsets, after acquisition of at least 50000 cells per PB sample, were identified using 2 approaches: 1) calculating the number of DCs when gated as lin-, HLA-DR+ and identifing the 2 subsets as CD11c+ (DC1) and CD123+ (DC2); 2) using three specific markers: BDCA.1 (CD11c+ high/CD123+ low, myeloid DCs); BDCA.2 (CD11c−/CD123+high, lymphoid DCs); BDCA.3 (CD11c+low/CD123−, myeloid DCs). Six parameter, 4-color FCM procedures were perfomed with a BD FACS Canto. Results: Our mean values of the percentage and of absolute number were: DCs ($0.5 \pm 0.2\%$; $30 \pm 11$ cells/μL); DC1 ($0.2 \pm 0.1\%$; $15 \pm 6$ cells/μL); DC2 ($0.2 \pm 0.1\%$; $15 \pm 7$ cells/μL) and DC1/DC2 ratio

(1.1 ± 0.5%); BDCA.1 (0.2 ± 0.1%; 16 ± 7 cells/μL); BDCA.2 (0.2 ± 0.1%; 12 ± 7 cells/μL); BDCA.3 (0.02 ± 0.01%; 2 ± 1 cells/μL). Conclusions: The results of BDCA.-subsets were similar to the that DC subsets confirming that the two type analysis identified the same DC populations. The FMC approach described is rapid and sensitive and can be utilized in the clinical setting as an additional indicator of the patients immunocompetence.

IN VITRO EFFECTS OF SHE-ASS MILK ON PERIPHERAL BLOOD LYMPHOCYTE RESPONSES

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She-ass milk (SM) is very close to human milk and, therefore, it is used in neonates when available. Additionally, in the case of bovine milk intolerance SM has safely been used as a good substitute. Here, we tested the effects of SM, taken at different intervals of time, on lymphocytes recovered from human healthy donors.

Ig production from B cells was evaluated by ELISPOT. Surface markers analysis of lymphomonocytes was assessed by cytofluorimetry.

Nitric oxide (NO) production was evaluated by spectrophotometry.

With regard to Ig production, only IgA but not IgG secretion was enhanced by samples of SM collected at 36 and 108 hours (h). Expression of CD69 on T cells was enhanced by samples of SM taken at 12 h and 22 days (D). Expression of CD25 on T cells was maximally expressed by SM at 213 D. Finally, NO production by monocytes was enhanced by samples taken at 211 D.

All together, these data indicate that SM may be a powerful immunomodulator in terms of IgA production, mostly at mucosal surface, of T cell activation and, finally, of NO production especially for its antimicrobial activity. Preliminary experiments also indicate that SM can represent a good supplement in the diet of aged people.

CD4+CD25+ AND CD3+V·24+ REGULATORY T CELLS IN HUMAN SOLID TUMOURS

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The immune response to tumour-specific antigens is typically unable to control the growth and spread of malignant cells. Accumulating evidence indicates that the effects of CD4+ CD25+ and natural T (NKT) lymphocyte populations are responsible for the failure of immune-mediated elimination of tumour cells.

Our study is addressing the analysis of such populations in the peripheral blood, as well as in metastatic nodes obtained from melanoma and in primary lung cancer and breast cancer lesions. Our preliminary data, obtained by comparative analysis of 51 melanoma patients with a group of 10 age/sex matched healthy controls, show a significant increase in the frequency of the NKT population in the peripheral blood of 11 melanoma patients (21.5%), while no significant variation in the CD4+CD25+ subset has been observed. All the patients with increased NKT population show a metastatic disease.

Besides sixty lung cancer patients have been analyzed. In lung cancinoma, our data show that the proportion of NKT cells in peripheral blood is significantly higher than in control group, while no significant variation in the CD4+CD25+ subset has been observed. Moreover, this study exams the relation between regulatory T cells and total tumour infiltrating T cell lymphocytes. We find a significant increase in the number of CD4+CD25+, CD4+CD25high and NKT cells in tumour as compared with the peripheral blood of patients with lung cancer. This could be referable to a mechanism of recruitment of this populations in tumour lesions. On the contrary, in breast cancer patients, the populations of CD4+CD25+ cells and NKT cells are not significantly higher than that in healthy volunteers with no significant variation respect to the peripheral blood.

These results suggest that NKT cells and CD4+CD25+/high cells may play a pivotal role in the anti-tumor immune response in lung cancer and in melanoma. The clinical and prognostic relevance of such data are under investigation.

## MICROSCOPY

IMMUNOFLUORESCENCE STUDY OF MYOTUBES DEVELOPMENT ON MICROGROOVED SURFACES

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Tissue engineering of skeletal muscle requires scaffolds that allow the contact guidance of cells because during tissue formation, skeletal muscle precursor cells fuse to form multi-nucleated myotubes. The formation of such structures requires the orientation and alignment of myoblasts in a correct structure prior to fusion. One method to affect cell behaviour is to obtain micrometer-scale features on substrate surfaces by means of photolithography. In this work we investigate the effects of different grooves dimension on cell guidance and myotubes formation. Microgrooved polymeric films were obtained by solvent casting (PLLATMC in chloroform) on microgrooved silicon wafers with different

grooves widths (5, 10, 25, 50, 100 μm) and depths (0.5, 1, 2.5, 5 μm) obtained by standard photolithographic techniques. In vitro static cell tests were performed using C2C12 murine myoblasts. Cell morphology was investigated by optical microscope and immunofluorescence assays labeling nuclei, actin and myosin. For all grooves depths, at 24 hours from seeding cells were aligned on films with 5, 10 and 25 μm grooves width. On films with 50 and 100μm grooves width no alignment and no preferential orientation were observed. Immunofluorescence microscopy of C2C12 cells grown on microgrooved surfaces 7 days after confluence showed the presence of elongated, multinucleated myotubes, they were differently oriented depending on grooves width and depth, while on smooth surfaces myotubes appeared randomly oriented.

Our results indicate that myoblasts can sense the microscale topographic features which can guide cell orientation, interaction and organization. Best results were observed for 2,5 and 1 μm depth especially for 50 and 25 μm width. For these films deeper investigations on myotubes development and myosin expression will be performed.

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## EXPLOITING NEW EXCITATION STRATEGIES IN FLUORESCENCE MICROSCOPY OPERATED BY LIGHT EMITTING DIODES

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The standard fluorescence microscopes are epi-fluorescence systems based either on 50W or 100W mercury arc lamps, the typical spectral emission showing some very bright narrow lines in both UV and visible spectrum. Despite their wide use, mercury arc lamps are not very efficient light sources for fluorescence microscopy. In fact, through a typical excitation filter, they usually provide between 0.5% and 3% of their total power suitable for sample excitation.

Since 2002 we had been pioneers in the application of Light Emitting Diodes (LEDs) as excitation device able to replace arc lamps in fluorescence microscopy. LEDs technology greatly improved in these last few years. The last generation of high power LEDs supplies up to 1 Watt of optical power within a very narrow spectral band. The peculiar characteristics of LEDs combined with compact plastic lenses made possible the design of an excitation device operating as "Abbe condenser" in transmitted illumination. This device can be applied to the conventional microscopes, up-grading them to fluorescence.. A further improvement has been done using a special design "water-immersion" Abbe condenser to achieve 50% more excitation intensity from the LED. Actually a new project based on the possibility to use three different diodes in a module give interesting horizons of fluorescence multi-excitation. An epifluorescence device has also been designed able to operate on inverted microscopes thus upgraded to fluorescence with the performances of LED illumination. A "twin-excitation" (combined epi and transmitted) fluorescence microscope is under development yielding a wide range of excitation performances able to offer the proper fluorescence illumination to any application requirements.

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## SHIM AND TPEM: GETTING MORE INFORMATION FROM NON LINEAR EXCITATION

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It's known that several endogenous protein structures give rise to second-harmonic generation (SHG) - second order nonabsorbative energy doubling of an excitation laser line [1].

In this work we look at several biological samples, bone, cartilage, tendon, tumoral tissue and zebrafish, where collagen or myosin proteins are present. We show how to use SHIM (Second-harmonic imaging microscopy) in combination with TPEM (Two Photon Excitation Microscopy) in a relative easy way. SHIM on a laser-scanning system is a powerful tool for high-resolution, high-contrast, three-dimensional studies of living cell and tissue architecture. The physical origin of SHG in tissues is addressed and attributed to the laser interaction with dipolar protein structures enhanced by the chirality of the protein helices. The multiple scattering through the tissue allows acquiring signal in both backward and forward direction [2]. SHG does not suffer photobleaching or toxicity and does not require exogenous labels. SHIM provides intrinsic confocality and deep sectioning in complex tissues. In this study, SHIM and two-photon excited fluorescence are combined in a dual-mode nonlinear microscopy to extract information from the specimen [3]. Both polarization and differences between forward and backward signals contribute to understand local structure.

**References**

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[2] A. Diaspro et al. Functional imaging of living Paramecium by means of confocal and two-photon excitation fluorescence microscopy, *Op. Diag. Liv. Cells* 5, 24 (2002).

[3] W.R. Zipfel et al. Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation, *PNAS* 12, 7075 (2003).

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## ROLE OF 3D BLEACH DISTRIBUTION in FRAP (FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING) EXPERIMENTS IN CONFOCAL AND TWO-PHOTON EXCITATION SCHEMES

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FRAP is a classical tool for quantitative evaluation of 2D diffusion processes. The spreading of confocal laser

scanning microscopes, together with the development of fluorescent proteins has encouraged the extension of this methodology to 3D environments. The quantitative analysis of such experiments requires the development of suitable analytical models capable to describe the experimental conditions. When diffusion in 3D is considered, the description of the initial condition produced by the perturbation (i.e. the photobleaching of a selected region) represent a crucial aspect, as the approximations that are usually made can lead to deviation in the measurement of the kinetic parameters of the labeled molecules. Furthermore the experimental distribution of fluorescent molecules depends on the intensity of the light pulse that produce the perturbation. In this work we measured the experimental 3D bleaching distributions produced in conventional and two-photon excitation schemes and analyzed the deviations from the idealized cases. The experimental measurement of these pattern for different experimental conditions in immobile samples (labeled polyelectrolyte gels) revealed that the approximation of the confocal bleaching intensity distribution as Gaussian can lead to relevant errors. On the opposite side the two-photon bleach volume seems well described by such approximation, even when fluorescence saturation effects arise.

These data has been used for finite elements simulations mimicking FRAP experiments on free diffusing molecules and compared with model FRAP curves based on the idealized bleach distributions. The results show that two photon excitation provide a better fit to the idealized bleaching patterns even in fluorescence saturation regime, resulting in correct estimations of diffusion coefficients within the 20%.

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IMMUNOFLUORESCENCE ANALYSIS OF SAOS-2 CELLS GROWN ONTO A SANDBLASTED AND PLASTICALLY DEFORMED TITANIUM SURFACE

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The titanium surfaces with micro-roughness have been studied to substitute machined titanium, with the focus on enhancing the bone apposition onto the implant [1]. We have followed a biomimetic strategy where human SAOS-2 osteoblasts proliferated and built extracellular matrix on a sandblasted titanium surface modified with plastic deformation. Our aim was to investigate the effects of the plastic deformation in terms of cellular proliferation and matrix production.

Cells were seeded onto sandblasted ("control culture") and punched-sandblasted titanium surfaces. The titanium surfaces were washed with phosphate buffer saline, fixed with formaldehyde, and processed for immunofluorescence detection of specific bone markers, such as type-I collagen, decorin, and osteopontin.

Immunofluorescence analysis showed that the plastic deformation, with the formation of "micro-wells" onto the rough titanium surface, improved the cell distribution on the titanium surface and caused significantly higher fluorescence intensity.

These data seem to suggest that the plastic deformation could be used to improve osteoblast growth and calcified matrix development in vitro.

**Reference**

[1] Martin JY, *et al.* J Biomed Mater Res 1995;29:389–401.

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IMMUNOFLUORESCENCE ANALYSIS OF SAOS-2 CELLS GROWN ONTO A TITANIUM PLASMA-SPRAY SURFACE

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The modification of a titanium surface plays an important role in bone tissue engineering. We have followed a biomimetic strategy where electromagnetically stimulated SAOS-2 osteoblasts proliferated and built extracellular matrix on a titanium plasma-spray surface. Moreover, increasing evidence suggests that an electromagnetic stimulus can modulate bone histogenesis and calcified matrix production in vitro and in vivo [1]. Our aim was to investigate the effects of an electromagnetic wave (intensity of magnetic field, 2 mT; frequency, 75 Hz) on human SAOS-2 cells in terms of proliferation and matrix production.

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

Immunofluorescence analysis revealed that the stimulation improved the cell distribution on the titanium surface and caused significantly higher fluorescence intensity.

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

**Reference**

[1] Heermeier K, *et al.* Bioelectromagnetics 1998;19(4): 222–231.

## SAOS-2 CELLS STIMULATED BY LOW POWER ULTRASOUNDS ONTO A TITANIUM PLASMA-SPRAY SURFACE

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In the present study we have followed a biomimetic strategy where ultrasonically stimulated SAOS-2 osteoblasts proliferated and built extracellular matrix on a titanium plasma-spray surface. There is increasing evidence that an ultrasound stimulus can modulate bone histogenesis and calcified matrix production in vivo [1]. Our aim was to investigate the effects of an ultrasound stimulus (power, 149 mW; frequency, 1.5 MHz) on human SAOS-2 cells in terms of proliferation and matrix production.

Cells were seeded onto titanium plasma-spray surfaces, and ultrasonically stimulated ("ultrasonic culture") or not ("control culture"). The titanium surfaces were washed with phosphate buffer saline, fixed with formaldehyde, and processed for immunofluorescence detection of specific bone markers, such as type-I collagen, decorin, and osteopontin.

Immunofluorescence analysis revealed that the stimulation improved the cell distribution on the titanium surface and caused significantly higher fluorescence intensity.

These data seem to suggest that the ultrasound stimulation could be used to improve osteoblast growth and calcified matrix development in vitro.

**Reference**

[1] Heckman JD, *et al.* J Bone Joint Surg Am (1994);76(1):26–34.

## IMMUNOHISTOCHEMICAL FMRFAMIDE-LIKE DISTRIBUTION IN THE CYPRID OF THE BARNACLE *BALANUS AMPHITRITE*

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The presence and distribution of FMRFamide-like peptides (FLPs) in the cyprid of the barnacle *Balanus amphitrite* were investigated by immunohistochemical methods. FLPs immunoreactive (IR) neuronal cell bodies were detected in both central and peripheral nervous system. In the brain three contralateral neuron somata were immunodetected and numerous IR nerve fibers in the neuropil area and optic lobes were observed. An intense immunostaining was also observed in the frontal filament complex: frontal filament tracts leaving the optic lobes and projecting towards the compound eyes, the swollen nerve endings in the frontal filament vesicles and the thin nerve endings in the external frontal filament. Thin IR nerve fibers were also present in the cement glands. Two pairs of neuronal cell bodies were instead immunodetected in the posterior ganglion; some of their axons seem to project to the cirri. FLPs IR neuronal cell bodies were also localized in the wall of the dilated mid-gut and in the narrow hindgut; their processes surround the gut wall and allow gut neurons to synapse each others. Our data demonstrated the presence of FLPs in the barnacle cyprid; their distribution allows us to hypothesise for these peptides an integrator role in the central nervous system, neuromuscular functions for thoracic limbs and intestinal movements and a neurosecretory role, exerted by the frontal filament complex, in the settlement and molting.

## IN-VITRO STUDY OF A NEW TREATMENT FOR CONTROLLING BACTERIAL ADHESION TO TITANIUM AND TITANIUM-ALLOY DENTAL IMPLANTS

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The development of new implant dentistry materials allowing to control bacterial adhesion and proliferation is an extremely topical issue in modern rehabilitation dentistry. Placing an osseointegrated implant may lead to the bacterial colonisation of the transmucosal portion of the implant, with the resulting progressive loss of contact between implant and soft tissues. As a result, there can be pathogen aggression of mineralised tissue, and impaired osseointegration, and even failure.

Different electrochemical methods for changing the implant surface, i.e. the thickness and structure of the passivity film investing titanium and titanium alloys were investigated and observed by Scanning Electron Microscopy (SEM). These techniques may enable to obtain a crystalline surface titanium oxide film, potentially capable of reducing bacterial colonisation in osseointegrated implants once positioned and loaded in the mouth. In particular, the influence of the anodisation voltage was investigated as applied to commercially pure, grade 2 titanium and Ti6Al4V surfaces, on the inhibition of the adhesion and proliferation process at 3 and 24 hours, respectively, for 4 bacterial strains: *Streptococcus mutans, Porphyromonas gengivalis, Staphylococcus aureus* 8325-4 and *Staphylococcus epidermidis* RP62A.

## MAP KINASE INTERACTS WITH TIGHT JUNCTION (TJ) DISRUPTION IN A HUMAN BRONCHIAL EPITHELIAL CELL LINE (BEAS-2B) BY CIGARETTE SMOKE (CS) EXPOSURE

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CS, the primary cause of chronic obstructive pulmonary disease, may damage epithelium integrity through the

disruption of TJs. The TJ integrity is regulated by G-protein-complex receptors (GPCRs) through transactivation of the epidermal growth factor receptor (EGFR) and subsequent activation of MAPK-ERK1/2 cascade. Using Beas-2B, we evaluated: a) the effect of CS (1.25–20%) after 24–72h incubation, on the cell morphology and TJs integrity, evaluated as the zonula occludin (ZO)-1 exp by immunofluorescence confocal microscopy; b) the possible involvement of EGFR/MAPK-ERK1/2 cascade following CS exposure by Western Blotting. CS induced a dose- and a time-dependent reduction of ZO-1 exp associated with a remarkable changes in cell shape suggesting a cell damage. CS (5%) was also able to phosphorylate ERK1/2 after 5 min and 4 h, and this effect was blocked by pre-treatment with EGFR inhibitor (AG1478, 1μM) and with a ERK1/2 inhibitor (U0126, 25μM). Since cAMP can interfere with MAPK pathway, Beas-2B cells were also pre-incubated with dibutyryl cAMP, a cAMP analog, resulting in a blockage of CS-induced ERK 1/2 phosphorylation. In addition, pre-treatment of Beas-2B with AG1478 or U0126 partially prevented CS-induced TJ disruption whereas exposure to a cAMP analog resulted in a complete redistribution of ZO-1 in TJ. These results suggest that TJ disruption induced by CS is mediated by EGFR-MAPK-ERK1/2 pathway and can be partially protected through activation of cAMP.

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γ-AMINOBUTYRIC ACID (GABA) RECEPTORS IN A CALCISPONGE

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Calcareous sponges are characterized by a primitive structural organization constituted by tube-like units with thin walls and lateral chambers enclosing a central cavity. Body walls are covered by a monolayer of esopinacocytes at the outer surface, while a monolayer of choanocytes lines the inner surface of the lateral chambers (choanocyte chambers). Although calcareous sponges display prompt reactions to both environmental and experimental stimuli, and are able of directional movements at the larval stages of their life cycle, the existence of an integrative system in these lower metazoans is till now hypothetical. Here we report the presence of GABA-like receptors in the calcisponge *Leuconia aspera* using immunofluorescence and confocal microscopy. Both $GABA_B$ R1 and R2 subunits were detected in the choanocytes lining the choanocyte chamber. Among the $GABA_A$ receptor subunits found in *L. aspera,* $\alpha_1$-, $\alpha_2$-, $\alpha_3$-, $\beta_{2/3}$- and $\gamma_2$-subunits are commonly expressed in neurones of the cerebral cortex, hippocampus, amygdale and olfactory bulb whereas $\alpha_6$-subunit appears to be restricted to cerebellar granule cells and dorsal cochlear nucleus. The involvement of GABA receptors in the endocytic processes of *L. aspera* was evidenced using a combination of pharmacological experiments with GABA receptor antagonists, agonists, and uptake inhibitors. These results indicate the existence of a complex intercellular communication system also in sponges with a very simple organization plan.

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INCREASED NEUTROPHIL (NEU) ADHESION IN BRONCHIAL BIOPSIES FROM PATIENTS WITH SEVERE COPD

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Increased Neu presence has been reported in COPD, particularly in sputum and BAL from mild/moderate patients and in bronchial biopsies from severe diseased patients. CXC and CC chemokines and their receptors are thought to play a role in Neu chemoattraction and activation. Furthermore, increased tissutal neutrophilia may be related also to increased adhesion to submucosal collagens in the submucosa.

We investigated the expression of CXCL7, CCL5, CD44 and CD11b in bronchial biopsies from subjects with severe COPD (n=13), mild/moderate COPD (n=12), and from control smokers (Ctr-S) (n=12) and control non smokers (Ctr-NS) (n=11). Immunopositivity was quantified as number of cells$^+$/mm$^2$ in the submucosa.

Severe COPD and mild/moderate COPD had higher CXCL7$^+$ in the submucosa (38(0–83) and 74(0–129)) compared to Ctr-NS (14(0–32)) but did not differ from Ctr-S (24(0–136). Severe COPD also had higher CCL5$^+$ in the submucosa (281(75–839) compared to Ctr-NS (77(26–203)). No differences were observed in the total cell count of CD44 and CD11b receptors in the four groups examined. Double staining of Neu coexpressing CD44 and CD11b showed increased percentages of CD44+Neu+ and CD11b+Neu+ cells in severe COPD (48(39–54) and 47(38–52)) compared to Ctr-S (13(9–20) and 17(10–21)). These data show that increased presence of chemotactic factors for Neu and increased Neu adhesiveness may play a role in sustaining neutrophilia in patients with severe COPD.

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PHOTODYNAMIC EFFECTS OF TOLUIDINE BLUE ON STAPHYLOCOCCAL BIOFILMS

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A significant proportion of medical implants are the cause for device related infections. Bacteria involved in pathogenesis related to implanted medical devices form biofilms adhering to substratum embedded in a self produced extracellular polymeric matrix. Staphylococci are the common bacteria that often form biofilms on medical

implants in hospital environment and are most intractable. Infections caused by these bacteria are difficult to treat due to their increased resistance to antibiotics. Various strategies are being exploited for eradication of biofilms. Photodynamic therapy (PDT) is an alternative approach for inactivation of antibiotic-resistant bacteria in which non-toxic photosensitizing dye and visible light are used for bactericidal activity.

We have investigated the effect of photodynamic action of toluidine blue O (TBO), a phenothiazine photosensitizing dye and diode laser (640 nm) on biofilms produced by *S. epidermidis RP-62A* and *S.aureus LP,* a methicillinresistant clinical isolate. Significant decrease in viability of cells was observed when staphylococcal biofilms were exposed to TBO and light simultaneously. The effect was found to be light dose-dependent. Confocal laser scanning microscopic study provided a direct evidence for changes in viability and structure of biofilms subjected to photodynamic treatment. The results suggest that photodynamic treatment could be a useful approach for inactivation of Staphylococcal biofilms.

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paGFP 3D LOCALIZED PHOTO-ACTIVATION AND TRACKING IN LIVING CELLS

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Photo-activatable Green Fluorescent Protein (paGFP) exhibits peculiar photo physical properties making it an invaluable tool for protein/cell tracking in living cells/organisms. paGFP is normally excited at near UV wavelength (405 nm), with an emission peak centered at 520 nm. Absorption cross section at 488 nm is low in the non-activated form. However, when irradiated with high-energy fluxes at 405 nm, the protein shows a dramatic change in its absorption spectra making it efficiently excitable at 488 nm. Total Internal Reflection Fluorescence Microscopy (TIRF) allows for localised 3D excitation of fluorescent molecules inside an evanescent electromagnetic field at interfaces such as cellular membranes. Optimization of the optical set up of an objective based TIRF system allowed us to demonstrate photoactivation of paGFP fused to different membrane localizing fusion proteins. Characterization of the penetration depth showed that activation is efficiently confined in the third dimension. Two-photon fluorescence microscopy removes the restriction of localization at interfaces providing optical confinement at any focal plane within the sample volume. We therefo-re characterized two-photon excitation and activation pro-perties of paGFP, showing that two-photon imaging and activation is feasible in the 750–820 nm range producing a narrow confinement along the optical axis. Optically confined photoactivation can produce novel insights into the study of biophysical mechanisms such as molecular diffusion in cellular compartments: application to EGF receptor mobility analysis provides example of advanta-ges stemming from TIRF and two photon spatially confined photolabeling procedure.

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EXPRESSION AND FUNCTION OF THE ''GLIAL'' GLYCINE TRANSPORTERS GLYT1 ON GABAERGIC NEURONS AND OF THE ''NEURONAL'' GLYT2 ON ASTROCYTES IN MOUSE SPINAL CORD

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Glycine transporters mediate glycine uptake both by astrocytes (GLYT1) and by glycinergic neurons (GLYT2). We here used purified synaptosomes and gliosomes of mouse spinal cord to characterize functionally and morphologically the glial vs. neuronal distribution of GLYT1 and GLYT2. Both gliosomes and synaptosomes accumulate [$^3$H]GABA through GAT1 transporters and, when exposed to glycine, they release the radioactive amino acid in a receptor-independent manner, due to glycine penetration through its selective transporters into GABA releasing particles. The glycine-evoked release of [$^3$H]GABA is exocytotic from synaptosomes but GAT1 carrier-mediated from gliosomes. Based on the sensitivity of the glycine effects to selective GLYT1 and GLYT2 blockers, surprisingly both transporters contribute equally to [$^3$H]GABA release from GABAergic synaptosomes, and the neuronal GLYT2 contributes more efficiently than the glial GLYT1 to mediate [$^3$H]GABA release from gliosomes. The anatomical significance of these functional results was largely explained by confocal microscopy: although gliosomes and synaptosomes preferentially express GLYT1 and GLYT2, respectively, GLYT1 are present on nerve endings and GLYT2 on gliosomes in a surprisingly remarkable amount. Moreover, co-localization analysis shows that the percentage of synaptosomes coexpressing GAT1 and GLYT2 is similar to the percentage of synaptosomes co-expressing GAT1 and GLYT1 and that the percentage of gliosomes co-expressing GAT1 and GLYT2 is higher than the percentage of gliosomes co-expressing GAT1 and GLYT1, in agreement with the functional data. To conclude, functional GLYT1 are present on neuronal axon terminals and functional GLYT2 on astrocytes, indicating not complete selectivity of glycine transporters in their glial vs. neuronal localization in the spinal cord.

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HIGH THROUGHPUT 3D CORRELATIVE MICROSCOPY

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Correlative microscopy allows solving several limitations of fluorescence microscopy, since the very same structures observed at the fluorescence light microscopy

(FLM) level can be analyzed as well by electron microscopy (EM). Therefore, it is possible to determine the real size and shape of structures observed by FLM and to solve the problem of absence of 'reference space': in FLM the unlabelled structures are not visible and do not contribute to the analysis while in EM all structures (labelled and unlabelled) are observable.

Unfortunately, these studies turned out to be extremely time consuming, and are not suitable for statistically relevant data.

In order to overcome these limitations we designed a new 3D correlative method, based on the use of semi-thin cryo-sections. Expected advantages are: hundred times more events that can be correlated in each single microscopy session; 3D correlation between FLM and EM; improvement of the resolution along the Z-axis, at FLM observation, due to the utilization of sections with a subresolution thickness.

This method has been applied successfully in linking studies between rough endoplasmic reticulum (RER) and endoplasmic reticulum-golgi intermediate compartment (ERGIC), two cellular compartments involved in the accumulation of immunoglobulins in Russel bodies [1].

**Reference**

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## ONCOLOGY

### CD133 AS A MARKER OF TUMORIGENIC CELLS FROM HUMAN SOLID CANCERS

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According to the cancer stem cell model, rare transformed cells asymmetrically proliferate to self-renew and produce daughter cells which can proliferate and partially mature to gain some features of the tissue of origin, similarly to their stem cells counterpart in tissues with high cell turnover such as the hematopoietic system. So called cancer stem cells have been found in solid cancers such as glioblastoma multiforme based on the expression of surface markers or on biochemical properties.

We have analyzed cells dissociated from primary human solid cancers either colon or lung, to identify and eventually isolate cells capable of reproducing the original tumors in animal models.

Colon cancer-derived epithelial cells contained a small number of CD133+ cells (2.5 $\pm$ 1.4%) that were sorted by FACS. Tumors became apparent in the injected animals at different times after injection depending on the number of cells injected. Unsorted cells from colon cancers induced tumor growth in 28% of the animals only when injected at $10^6$ cells/site. CD133+ cells generated tumors in 56% of the animals by injecting $5 \times 10^3$ cells/site. No detectable growth of cancers cells was observed in mice injected with CD133- cells. Due to the low number of CD133+ present in freshly dissociated lung cancer cell suspensions, only unsorted cells were injected in this case and tumors were produced in about 10% of the mice. Cells from the tumors developed in animals were enriched in CD133+ cells and retained the ability to induce tumors after several consecutive transplantations. We conclude that CD133 is associated with the capability of transformed cell in human solid cancers to regenerate tumors in experimental models.

### INSULIN-LIKE GROWTH FACTOR 1 AND ESTRADIOL REGULATES ESTROGEN RECEPTOR-DEPENDENT TRANSCRIPTION AT AP-1 SITES IN BREAST CANCER CELLS

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IGF-1 is known to stimulate AP-1 activity in breast cancer cells and to stimulate cyclin D1 transcription and enhance cyclin D1 mRNA stability and protein levels. Cross-talk between insulin-like growth factor 1 (IGF-1) and estradiol (E2) pathways can regulate the cyclin D1 activity in breast cancer cells, but the underlying mechanisms remain unclear. IGF-1-induced cyclin D1 expression in MCF-7 breast cancer cells depends on the presence of ER. Here, we studied how 17-b-estradiol (E2) and IGF-1 affect ER transcriptional machinery in MCF-7 cells. In this study, we explored using in vitro and in vivo techniques how E2, IGF1 and the combination of both factors regulate ER nuclear translocation, ER recruitment to AP-1 motifs cyclin D1, and ER complex assembly at this site. E2 treatment stimulated ER loading on the AP-1 motif in the cyclin D1 promoter, ER binding fluctuated over time (1–24h). Under IGF stimulation liganded ER was recruited to AP-1 site together with histone acetyltransferases SRC-1, p300, ubiquitin ligase E6-AP, mdm2 and polymerase (pol) II. This coincides with high expression of mRNA level of Cyclin D1. E2 moderately increased cyclin D1 expression, which was associated with the recruitment of liganded ER, SRC-1, p300, ubiquitin ligase E6-AP (E6L), Mdm2, and pol II, but not other regulatory proteins, to AP-1. Notably, ER knockdown reduced the association of ER, E6L, Mdm2, Carm, and pol II with AP-1 and resulted in down-regulation of cyclin D1 expression. IGF-1 did not potentiate the effects of E2 on AP-1 site and did not increase mRNA cyclin D1 expression.

## SYNERGISTIC EFFECT OF HYPOXIA AND INSULIN ON LEPTIN EXPRESSION: ROLE OF HYPOXIA-INDUCIBLE FACTOR-1α IN BREAST CANCER CELLS

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We reported previously that the obesity hormone leptin is overexpressed in primary and metastatic breast cancer relative to non-cancer tissues. Here we investigated molecular mechanisms of leptin expression in breast cancer focusing on the effects of obesity-related stimuli such as hyperinsulinemia and hypoxia.

Using quantitative real-time PCR, immunofluorescent detection of proteins and ELISA assays, we found that treatment of MCF-7 breast cancer cells with high doses of insulin and/or hypoxia conditions significantly increased the expression of leptin mRNA and protein. Notably, the greatest leptin mRNA and protein expression was observed under combined hyperisulinemia and hypoxia conditions.

These treatments also increased nuclear expression of hypoxia-inducible factor 1α (HIF-1α) and increased its interaction with several hypoxia responsive elements (HREs) in the leptin promoter, especially with the proximal promoter containing four hypoxia response elements and three GC-rich regions.

The HIF-1α complex interacting with the proximal promoter included p300 histone acetyltransferase, the major HIF coactivator. Induction of HIF-1α binding to the leptin promoter was paralleled by the increased leptin mRNA and protein expression. On the other hand, HIF-1α knockdown with RNA interference totally abolished leptin expression in breast cancer cells.

Also in this case, the greatest accumulation of nuclear HIF-1α, the highest association of HIF-1α and p300 binding with the proximal leptin promoter, were induced by combined insulin and hypoxia treatments.

It is important to note that HIF-1α is often overexpressed in invasive breast cancer tissues, and HIF-1 expression is also a predictive marker of chemotherapy failure. Thus, molecular targeting of HIF-1α might help in the treatment of leptin-overexpressing cancers.

## CEREBROSPINAL FLUID FLOW CYTOMETRY IMMUNOPHENOTYPING GREATLY IMPROVES DIAGNOSIS AND MONITORING OF TUMOUR INFILTRATION

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Neoplastic meningitis (NM) is a common problem in neuro-oncology occurring in approximately 5% of all cancer patient. Morphological evaluation of cerebrospinal fluid (CSF) by light microscopy is the diagnostic "gold standard" for the detection of central nervous system involvement in patients with tumour meningitis; however, it is a low sensitive method, with a reported false-negative rate of 20% to 60%.

Flow cytometry immunophenotyping (IF) is an essential tool for the diagnosis and monitoring of haematological malignancies in routine clinical practice and is increasingly utilised to detect haematological CSF infiltration.

In this study we assessed the value of six colour flow cytometry IF in 36 CSF sample from patient with different type of cancers: 8 Diffuse Large B Cell Lymphoma, 3 multiple myeloma (MM), 3 acute leukaemias, 10 breast, 1 bladder, 2 lung and 9 brain carcinomas.

Despite the low absolute cell number (8 cell / μl, range 1–182), a characterization of >500 valuable events was obtained in all samples. A proportion of CD38 CD138 CD28 CD117 CD56 Kappa positive plasma cells was identified in 3 MM cases; a median of 29% (range 1–85) CD45 negative tumour cells was observed in breast (n=5), lung (n=2), bladder (n=1) neoplastic meningitis and in 2 brain tumours. No false positive cases were observed. Three flow cytometry positive cases where negative by conventional cytology.

When combined with cytology, flow cytometry CSF IF can increase detection sensitivity for the diagnosis and monitoring of NM, particularly useful in low CSF cell numbers cases.

## FLOW CYTOMETRY IMMUNOPHENOTYPING OF PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA: A NOVEL DIAGNOSTIC APPROACH TO STEREOTACTIC BIOPSY

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Primary central nervous system lymphoma (PCNSL) is a rare haematological disease, with a constant increasing incidence in recent years, up to 6% of primary intracranial tumours in different neuropathological series. The incidence rises up to 25% when only multiple brain lesions have been considered. Neurosurgical removal of PCNSL has any relevant therapeutic role and does not increase the survival, being chemo and radiotherapy the mainstays of therapeutic strategy. Brain bioptic sampling remains the gold standard for PCNSL diagnosis in all patients, and stereotactic biopsy (SB) is the method of choice, allowing biopsy of deep-sited lesions that could not be approached safely with

conventional open surgery. However, SB could be inconclusive in a limited number of cases (10–15% of cases), mainly after high dose steroid treatment, and a second diagnostic biopsy is necessary at the moment of clinico-radiological recurrence.

Flow cytometry immunophenotyping (IF) is an indispensable tool for the diagnosis and monitoring of haematological malignancies in routine clinical practice, however it has not been reported for the diagnosis of SB in PCNSL. In this study, in additional to conventional histopathology, we assessed the value of six colour flow cytometry IF in SB biopsy of 2 PCNSL cases. After tissue dissociation a single cell suspension was obtaining for flow cytometry analysis. Diagnostic characterization of the leukocyte population was performed, with a number of >15.000 valuable events, in both samples. A proportion of CD45 CD19 CD20 CD22 positive, CD79b CD5 CD10 CD34 negative large cells was identified, allowing the diagnosis of large B cell lymphoma in both cases. A significant infiltration of T CD2 CD3 CD5 CD8 positive lymphocytes was observed, in proportion of 10% and 40% of the CD45 leukocyte population, respectively.

Flow cytometry IF of stereotactic biopsy appears a new powerful, reliable and rapid technique for the diagnosis of PCNSL and can improve the diagnostic approach and therapeutic strategies of brain tumours.

## ASTROCYTOMA GAP JUNCTION INTERCELLULAR COMMUNICATION (GJIC) IS MODULATED BY X-RAY ACTIVATED MICROGLIA

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Brain tumors are characterized by a reactive gliosis involving normal astrocytes and microglia. Brain inflammation is mainly accompanied by glial cell activation and expression of proinflammatory cytokines. Moreover Interleukin1, (IL-1β,) and Tumor Necrosis Factor-α (TNF-α), produced by microglia, have been identified as the main factors responsible for GJIC inhibition in astrocytes. Radiotherapy represents a standard clinical management of infiltrating high-grade astrocytomas and such treatment usually involves normal surrounding cells in order to maintain an adequate "safety margin". Since disorders of GJIC are associated with aberrant cell growth, the aim of this study was to investigate the role of surrounding normal microglial irradiated cells in modulating GJIC of astrocytoma cells. To address this issue, we used BV-2 murine immortalized microglial cell line and C6 rat astrocytoma cells, analysed with a quantitative cytofluorimetric assay for GJIC determination. Conditioned medium of BV-2 cells treated with X rays is sufficient to induce the inhibition of astrocytoma GJIC in a dose-response manner. These results suggest a possible role of surrounding radiation-induced proinflammatory cytokines in modulating astrocytoma GJIC.

## CHROMOSOME 17 ANEUSOMY AND HER-2 GENE COPY NUMBER IN 2+ IHC BREAST CANCER

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Laboratory assessment of HER-2 status is becoming a key step in the optimal management of patients with advanced breast cancer. Aberrant numerical changes in chromosome 17 copy number are frequently encountered in invasive breast cancer and may complicate the scoring of HER-2 amplification.

Our aim was to determine the aneusomy level and the HER-2 gene copy numbers, by fluorescence in situ hybridization (FISH) and to analyze their impact on the amplification rate in breast carcinomas considered HER-2 weakly positive cases by immunohistochemistry. We evaluated 343 breast carcinomas using double colour FISH (LSI Her-2/neu gene and CEP 17). Monosomy and polysomy were demonstrated in 24.2% and 46.1% respectively and 101/343 (29.6%) of the specimens resulted amplified by FISH. A statistically significant difference was observed when we compared the amplification percentage in polysomic and monosomic specimens ($p < 0.0001$) and, among polysomic specimens, when tumours were compared with HER-2 gene signals number per cell between 3 and 10 and >10 respectively ($p < 0.0001$). Logistic regression analysis showed that HER-2 signals >10 and polysomy absence were independently associated with amplification. Our results confirm that the majority of 2+ IHC cases express the HER-2 protein without gene amplification and highlight the effect of chromosome 17 aneusomy and the HER-2 gene copy number on amplification.

## FUNCTIONAL ASSAYS TO TEST CXCR4-INTERACTING NEW SYNTHESIZED PEPTIDIC STRUCTURES

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The overexpression of the chemokine receptors CXCR4 has been reported in several neoplasias confirming a role for the axis CXCR4-CXCL12 in tumor cell growth and metastases. Several CXCR4 inhibitors were described but none has reached the safety and effectiveness to be suitable for clinical use. In order to find effective and clinically suitable CXCR4 inhibitors several peptidic structures were designed on a rationale-based design and synthesized. 14 peptides were tested for CXCR4 functional interactions. The peptides were tested for the capacity of interfering with the specific chemokine (CXCL12) induction of P-Erk and with the specific chemokine (CXCL12) induced migration. In addition

association and dissociation was measured indirectly by flow cytometry using a PE-labelled anti CXCR4, 12G5 antibody. CCFR-CEM cells naturally expressed CXCR4, CCR4 and CCR7. The cells were incubated with 10, 1, 0.1 μM of AMD3100, a well known CXCR4 inhibitor, and then labelled with an anti-CXCR4-PE antibody and binding assessed by flow cytometry. The effect of our new synthetized peptides were compared to the effect of AMD3100. At least two compounds were identified that interfere with the P-Erk induction, migration and binding capacity such as CXCR4 inhibitors. This is an integrated new and promising approach to functionally validate CXCR4 inhibitors.

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CHROMOSOME X POLYSOMY, 8 ANEUSOMY AND LPL GENE DELETION: VALIDATION OF AN ADVERSE PROGNOSTIC PROFILE IN CLINICALLY LOCALIZED PROSTATE CARCINOMA

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We genetically characterized 106 prostate cancer patients by fluorescence in situ hybridization (FISH) in retrospective study. Probes for LPL (8p22), c-MYC (8q24) genes and for 7, 8, X chromosomes were used. Chromosomes 7, 8, X aneusomy was respectively demonstrated in 91.5%, 78.3%, 51.9% of the samples, whereas LPL deletion and MYC amplification were found in 76.0% and 1.6%. A genetic profile was considered as unfavorable when at least two chromosomes and one altered gene were present. The number of tumors, with an adverse genetic profile, statistically increased at higher stages ($p=0.02$) and gradually in patients with biochemical and clinical progression ($p=0.03$). In the Gleason score 7 group, the percentage significantly advanced in tumors with grade 4+3 ($p=0.02$). Multiple correspondence analysis identified one tumor group characterized by chromosome 8 aneusomy, X polysomy, LPL gene deletion, Gleason >7 and 4+3 associated with progression. The availability of biological profiles with different prognosis will help to obtain a more effective cure and more selective treatments tailored to the single patient can be hypothesized.

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FLOW CYTOMETRIC ANALYSIS OF THE EFFECTS OF IMATINIB MESYLATE (STI571) IN T98G GLIOBLASTOMA CELL LINE

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Glioblastoma, the most common of malignant brain tumours in adults, is resistant to all forms of the therapy, causing the death of most patients within 9–12 months after diagnosis. Therefore the search of improved treatment is urgently needed.

Many studies have indicated that in glioblastoma there is a coexpression of platelet-derived growth factor (PDGF) and its receptor (PDGFR), suggesting a role of this signaling pathway in the growth of these tumours. In this study, a T98G human glioblastoma cell line was analysed for its sensitivity to treatment with a selective PDGF receptor inhibitor, imatinib mesylate (STI571, kindly provided by Dr E. Alessandrino). In particular, we have focused our attention on the analysis of DNA distribution by flow cytometry at different times of incubation and concentrations (1–30 μM) of imatinib mesylate. Our results show that in T98G cells STI571 induces a growth arrest in the $G_0/G_1$ phase of the cell cycle at all concentrations tested already 24 hours after imatinib treatment. Moreover we have seen, with annexin V staining, that after 48 hours at 20 and 30 μM concentrations, in concomitance with a growth arrest in the $G_0/G_1$ phase, there is a significant increase of apoptotic cells, suggesting that at low concentrations STI571 could acts as a cytostatic agent but at high concentration it behaves mainly as a cytotoxic agent.

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PLATELET-DERIVED GROWTH FACTOR (PDGF) INCREASES IN VITRO PROLIFERATION OF HUMAN ASTROCYTOMA CELLS

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PDGFr is a growth factor receptor with intrinsic tyrosine kinase activity that is deregulated in several human diseases, including tumours. In astrocytoma an increased expression of PDGF and its receptor has been described, suggesting that autocrine and paracrine mechanisms of activation of this signalling pathway might play a role in astrocytoma cell proliferation.

In this study, we have described immunocytochemical PDGFRa expression in astrocytoma cell lines and we have focused our attention on its immunocytochemical expression in relation to its functional capacity, measuring the proliferative response induced by exogenous PDGF.

The analysis of PDGFRa expression in astrocytoma cell lines showed a grade-dependent staining intensity and localization. Interestingly, we observed a correlation between the proliferative response of astrocytoma cell to PDGF stimulation and PDGFRa immunocytochemical localization. In fact, the intensity and the timing of the response is related to the receptor status before PDGF stimulation, in particular, to the down-regulated status. Only in one of the astrocytoma cell lines studied (PRT-Hu2; grade IV) we observed a time-dependent response without an evident down-regulation of the receptor. The flow cytometry studies have showed that this cell line is heterogeneus; therefore the overall PDGF effect observed might be the sum of the effects induced in each cell population related to their PDGF sensitivity.

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INHIBITION OF POLY (ADP-RIBOSE) POLYMERASE SYNERGIZES WITH THE DNA-TOPOISOMERASE-1-INHIBITOR, TOPOTECAN, AND RADIATION IN HUMAN GLIOBLASTOMA CELL LINES

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Poly (ADP-Ribose) polymerase-1 (PARP-1) inhibition increases the citotoxicity of DNA-damaging agents and

ionizing radiation (IR) in several tumor models. We investigated the effects of combined treatment with the DNA-topoisomerase-1-inhibitor, topotecan (TPT), the PARP-1 inhibitor NU1025 and IR in both D54 $p53^{wt}$ and U251$p53^{mut}$ human glioblastoma cell lines. Cell growth studies showed that non-cytotoxic doses of NU 1025 (10μM) synergize with IR and TPT in both U251$p53^{mut}$ and D54 $p53^{wt}$ cells. In U251 $p53^{mut}$ cells, 10 nM of TPT induced 40% of cell growth inhibition after 96 hrs of treatment, than 2 Gy of IR about 20% only. The addiction of NU1025 synergize with both TPT (90% cell growth inhibition) and IR (45% of growth inhibition). The radiosensitive D54 $p53^{wt}$ cell line was strongly inhibited by 2Gy of IR (50% of growth inhibition) and about 30% by 10 nM of TPT. Even in this case, the addiction of NU1025 synergize with both IR (73% of growth inhibition) and TPT (62% of growth inhibition). DNA-flow cytometry did not show any specific perturbation of D54 $p53^{wt}$ cell cycle, than a $G_2$/M block and apoptosis were evident in U251 $p53^{mut}$ cells. Our findings suggest that the inhibition of PARP-1 activity could be considered a strategy to increase the specific effects of TOPO-1 poisons and RT in human glioblastoma cells a part from the p53 status.

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PHARMACODYNAMICS OF TRABECTEDIN ON INFLAMMATORY PATHWAYS

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The marine natural product Trabectedin (ET-743, Yondelis) was found to be active in refractory soft tissue sarcoma. Trabectedin binds in the minor groove of DNA, affects transcription regulation in a promoter dependent fashion and blocks cell cycle. In addition we have recently demonstrated that Trabectedin is selectively cytotoxic to monocytes/macrophages, being active at concentration that spared lymphocytes. We tested the effect of subcytotoxic concentration of Trabectedin on the production of inflammatory mediators.

Trabectedin dose-dependently inhibited the release of chemokines by monocytes and differentiated macrophages. Trabectedin also reduced CCL2 production by sarcoma cell line as 402-91 kindly provided by P. Aman, indicating that the antitumor activity could be partially related to an anti-flammatory effect.

The clinical observation of high activity of Trabectedin in myxoid liposarcomas has prompted us to investigate the drug mode of action in this tumor. The sensitivity of 402-91 cell line to Trabectedin was in the nanomolar range whereas flow cytometry analysis showed a delay progression in S phase and a block in G2M phase.

Since this cell line costitutively expresses pentraxin3 (PTX3), a protein whose role is important in the inflammatory response we have investigated whether Trabectedin was modulating this production. Trabectedin caused a dose-dependent reduction of PTX3 levels in 402-91 cell lines. When this cell line was incubated with human monocytes the production of PTX3 was increased by approximately three times. By treating both 402-91 cells and monocytes with as low as 0.25 nM Trabectedin, PTX3 expression was reduced by 75%.

Studies are in progress to evaluated the molecular mechanism underlying the observed effect and to investigate the relevance of these findings for the antitumor activity of Trabectedin

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ANTIPROLIFERATIVE AND CYTOKINETIC EFFECTS OF THE SPECIFIC AKT INHIBITOR PERIFOSINE ON DIFFERENT SOLID TUMOR CELL LINES

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Perifosine, a.k.a. KRX-0401 [octadecyl-(1,1-dimethylpiperidinio-4-yl)-phoshate] is a synthetic novel alkylphospholipid, member of a new class of antitumor agents which target cell membranes, inhibit Akt activation and induce apoptosis. Perifosine inhibits Akt activation regardless of the interaction ligand/receptor tyrosine kinase because it is able to exert its effects directly on the activated form of Akt; furthermore, Perifosine does not directly affect activity of PI3-K or phosphoinositide-dependent kinase 1 (PDK1) and induces $p21^{cip1}$ expression leading to G2/M phase cell accumulation. $p21^{cip1}$ induction is due to activation of Erk signalling pathway by Perifosine, since Erk activation promotes the phosphorylation of Sp1 in known Erk threonine residues (Thr 453/739), thereby leading to increased Sp1 binding and enhanced $p21^{cip1}$ transcription.

In this study, we have investigated the antiproliferative and cytokinetic effects of Perifosine on two renal (CAKI-2 and ACHN), on one bladder (HT1197) and on three different malignant mesothelioma (MMe) (MSTO-211H, MMB, REN) cancer cell lines in vitro, as well as on a tubular proximal renal epithelium cell line (HK-2) and on a normal mesothelial cell line (MeT-5A), used as a control. As far as the inhibition of cell proliferation, evaluated at 24 h using a Coulter (Model Z1, Instrumentation Laboratory), the resulting $IC_{50}$ values were: 0.05–0.5 μM for CAKI-2, 5 μM for ACHN, 1 μM for HT1197 and 7 μM for HK-2 cells. For

malignant mesothelioma and mesothelial cell lines, the resulting $IC_{50}$ values were: between 5–10 μM for MSTO-211H, 10 μM for MMB, and between 15–20 μM for both REN and MeT-5A cell lines. As far as Perifosine induced cytotoxicity, evaluated using the MTT method, the resulting $IC_{50}$ values were: 0.3–0.5 μM for CAKI-2, 3–5 μM for ACHN, 20 μM for HT1197, and between 3–5 μM for HK-2. For malignant mesothelioma and mesothelial cell lines the resulting $IC_{50}$ values were: between 10–15 μM for MSTO-211H, 15–20 μM for REN, 50 μM for MMB, and between 25–30 μM for MeT-5A. Double staining with propidium iodide and fluoresceine isothyocianate showed the presence of many polyploid and apoptotic cells in renal, bladder and malignant mesothelioma (MMe) cell lines. These results were confirmed by DNA flow cytometry of treated cells as compared to the controls. Perifosine-induced cytokinetic effects showed cell accumulation in the G2/M phase of the cell cycle and DNA fragments in the sub G0/G1 region of the DNA histograms, thus indicating apoptosis. Western blot analysis confirmed the role of the Perifosine as a selective inhibitor of Akt activation. Indeed, in the ACHN and HT1197 cell lines, a decrease of p-Akt1/2/3 compared to controls were observed, after 30′, at the concentration of 5 μM; in the CAKI-2 and in the HK-2, the same decrease was evident after 24 h. As far as p-Erk, it was expressed in the four cell lines at baseline and was not affected by the treatment with Perifosine, thus demonstrating that the drug did not affect the MAPK pathway.

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CD133(+) STEM CELLS IN A CELL LINE OF HUMAN OSTEOSARCOMA (SAOS2)

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BACKGROUND: Osteosarcomas are the most common tumors of bone and they are characterized by an elevated incidence among young adults with a fatal prognosis due to an early metastatization. The presence of stem cells in solid tumors has better clarified the mechanisms that regulate tumor growth and invasion with particular emphasis towards clinical settings aimed at targeting specific subpopulations of tumoral cells. Some evidence has suggested that CD133 is a marker for a subset of glioblastoma, melanoma, liver and colon cancer stem cells. We have firstly verified the presence of CD133+ subpopulations in human osteasarcoma cell line and subsequently assessed its stem-cell-like characteristics. RESULTS: Flow citometric analysis revealed that the percentage of the CD133(+) subpopulation was between the 2% to the 5% of cells; the analysis of the cycle with PI and Hoechst showed a clean difference in the cell-cycle progression between CD133(+) and CD133(−) cells. More specifically, CD133(+) resulted to be almost totally in phase G2\M whereas the CD133(−) resulted to be only around 15% of cells. The same result was confirmed utilizing the Ki67 marker along with the CD133. Finally, the growth in semisolid medium revealed that only CD133(+) cells were able to grow in suspension in the form of sphere while CD133(−) were not. CONCLUSIONS: Our data confirm the presence of a CD133+ stem-cell-like subpopulation in human osteasarcomas. Strategies aimed at identifying these cells will help to understand the key molecular mechanisms in tumor growth and progression.