Pre-Print of

PROCEEDINGS OF THE XXII NATIONAL CONFERENCE OF THE ITALIAN SOCIETY OF CYTOMETRY

GIC

San Benedetto del Tronto, Italy
Palazzo dei Congressi e della Cultura
October 4–8, 2005

Editors

R. De Vita – G. Mazzini

Società Italiana di Citometria

La Conferenza è organizzata dalla Società Italiana di Citometria GIC

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SOCIETÁ ITALIANA DI CITOMETRIA

XXII CONFERENZA NAZIONALE DI CITOMETRIA

PALAZZO DEI CONGRESSI E DELLA CULTURA, SAN BENEDETTO DEL TRONTO 4-8 OTTOBRE 2005

PRE-PRINT OF
PROCEEDINGS OF THE XXII NATIONAL MEETING

EDITED BY

R. DE VITA and G. MAZZINI

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XXII National Conference of the Italian Society of Cytometry GIC
October 4–8, 2005
San Benedetto del Tronto (AP), Italy

Guest Editors:
R. De Vita – G. Mazzini

This issue of Cytometry is partly dedicated to the programme and abstracts of the XXII National Conference of the Italian Society of Cytometry, GIC, held in October 2005 in San Benedetto del Tronto (Ascoli Piceno, Italy) and organized on a two-annual basis by the same Society.

The GIC was founded in Rome in 1986 by scientists involved in various field of the (at that time emerging as new technology in biology and medicine) flow cytometry. To date there are over 805 members. Among its many activities the GIC is involved in educational programs, scientific meeting organization, promotion of quality controls programs and drafting/validation of guidelines, providing information for people that actively work in the field of basic and applied cytometry. Other activities of the GIC are performed through workgroups and committees, addressed to the following principal objectives:

- The creation and implementation of guidelines concerning the major aspects of the various cytometric applications.
- The organization of a comprehensive educational programme, through the bi-annual Conference, scientific "theme-addressed" Conferences, inter-regional Courses and especially through the affiliated permanent School of Cytometry; all these activities are conducted within the framework of the Continuous Medical Education (CME) approach planned by the Italian Ministry of Health.
- The analysis and promotion of managerial quality in the discipline.

119 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 and updated report of the status of translational and clinical research in the field of cytometry in Italy.

Many investigators have submitted high quality data that were organized (as 49 oral and 70 poster presentation) inside specific sessions. Each session involved invited speakers and was focused on the emerging role of cytometry techniques in Environmental Sciences and Toxicology, Hematology, Immunology and Oncology.

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.

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

This scientific event is growing each year, and represent Italian cytometry's scientific contribution to the international scientific community.

Marco Danova
GIC President

CELL CYCLE AND APOPTOSIS

1

THE NORMAL – NEOPLASTIC SEQUENCE IN THE GASTROINTESTINAL TRACT: PAST AND UPDATED METHODS

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Introduction: Referring to Stomach cancer and Colon adenomas, the progression from normal to neoplastic lesions was investigated. The aim consisted of matching previous techniques with up dated methods, to find whether a correlation exists or not. **Methods**: Historical immuno-histochemical mucin techniques include: Periodic acid Schiff technique, Alcian blue staining and High iron diamine technique (HID). Evaluation of the secretory component on PAS, Alcian blue, HID staining, was associated to the detection of proliferative activity (Mib1- an immunohistochemical technique) and DNA flow cytometric content. **Results and conclusions**: Colon adenomas with mild-to-intermediate (70%) and severe (30%) dysplasia showed a progressive depletion of sialomucins (alcian blue +) and an increased proliferative activity (Mib1+). A topografic correlation between the two findings was elicited. DNA aneuploidy was observed in a significant number of adenomas which were marked by the sialomucin depletion. The frequent expression of sulfomucins (HID+) was a reliable index of intestinal metaplasia adjacent to Gastric cancer. Thus, this histochemical feature could predict a worse prognosis in intestinal metaplasia. Moreover, proliferative activity was found to be negatively related to secretory component. In order to check the progression of pre-cancerous lesions, the study of the secretory component with detection of sialo- and sulfomucin content is worth to be added to more recent markers (Mib1 and DNA Ploidy).

2

MITOCHONDRIA BEHAVIOUR DURING UVB-INDUCED APOPTOSIS AND MELATONIN PROTECTION

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Mitochondria can be easily affected by free radicals: protection of mitochondria against oxidative damage becomes increasingly important, playing an important role in the apoptotic mechanism. UV-B irradiation alters mitochondrial function causing a release of pro-apoptotic molecules, like cytochrome C (Cyt C). This last one acts as a trigger for the formation of a complex including the APAF-1 and procaspase-9, mediated by reactive oxygen species (ROS) that modify the mitochondrial membrane permeability. Being well known that melatonin (MLT) is a scavenger of ROS, we have investigated mitochondria behaviour in UV-B irradiated and MLT treated U937 cells. In our model, the treatment with 1 mM of MLT before UVB irradiation showed a significant protection from apoptotic cell death with respect to that found in UV-B exposed U937 cells; on the other hand, the treatment with MLT after UV-B did not show a significant protective effect. In particular, mitochondrial structure and function were preserved when U937 cells were incubated with MLT before UV-B exposure, as demonstrated by peculiar fluorescence of both MitoTracker Green FM (MT) and JC-1 markers. The treatment with MLT before UV-B exposure does not only protect the mitochondrial membranes (as revealed by MT staining) but we demonstrated that MLT may also stabilize the mitochondrial membrane potential $\Delta\psi$, inhibiting the release of Cyt C from the mitochondria into cytosol. The attenuation of Cyt C release, by MLT pretreatment, may significantly limits the apoptotic machinery induced by UV-B exposure, through the reduction of Apaf-1 and caspase-9 pathways. Our observations suggest that mitochondrial function and structure can be protected by MLT addition and that this neuro-hormone may play a key role in inhibiting apoptosis.

3

ANALYSIS OF BrdU INCORPORATION BY FLOW CYTOMETRY CAN BE MINIATURIZED IN 96 WELLS-PLATE FORMAT ALLOWING ACCURATE CELL CYCLE ANALYSIS

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BrdU incorporation is commonly used to evaluate the mode of action of many anticancer drugs. Usually relatively large amounts of cells and antibodies are required. Also sample handling is not suitable for high sample flux. To increase productivity and sample throughput, reducing time, compounds and amount of antibodies, we have developed a miniaturized method to measure BrdU incorporation and DNA content directly in 96-wells plates. Briefly human cancer cells were grown in presence of testing compounds in 96 well plates for 24 hrs. One hour before the harvest cells were exposed to 30 μM BrdU. Then cells were washed, detached by trypsin and transfered to PCR V-bottom plates. At the end cells were centrifuged, fixed by methanol 70% and stored

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overnight at 4°C. BrdU detection was performed directly in plates; DNA denaturation was performed by heat (95°C for 5 min) in an appropriate denaturation buffer using a 96 wells PCR thermocycler. BrdU incorporation was detected by indirect immunocytochemical staining using an anti-BrdU antibody followed by a FITC-anti mouse secondary antibody. Cellular DNA was counterstained by propidium iodide for DNA content. Samples were acquired by a BD FACSCalibur with BD Multiwell Autosampler (MAS) and analyzed by BD CellQuest and Modfit software. This method provided accurate and reproducible BrdU/DNA content analysis in different cell lines, reducing time and reagent consumption. A critical step is the standardization of DNA denaturation using a PCR Thermocycler. BrdU dot plots of cell cycle phases were well separated and DNA histograms showed lower CV%. Here we show some applications of this method for the evaluation of cell cycle effects in studies of mechanism of action by anticancer drugs treatments.

4

DYNAMICS OF FLUORESCENT PROTEIN-TAGGED p21CDKN1A AND PCNA RECRUITMENT TO DNA REPAIR SITES

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The cyclin-dependent kinase (CDK) inhibitor p21CDKN1A (also known as p21WAF1/CIP1/SDI1) is required for cell cycle arrest involved in checkpoint response to DNA damage, senescence, and terminal differentiation. p21 induces cell cycle arrest by CDK inhibition, and through the interaction with proliferating cell nuclear antigen (PCNA), thus inhibiting DNA replication. However, the role of this interaction during DNA repair is unclear. We have analyzed the dynamic relocation of p21 protein after DNA damage, and investigated the interaction with PCNA during DNA repair. HeLa cells were co-transfected with vectors, inducing the expression of p21-GFP and PCNA-RFP fusion proteins. After UV-C exposure, through filter with 3-μm pores, the recruitment of both proteins to DNA repair sites was determined by immunofluorescence, confocal microscopy, and western blot analysis. Early recruitment of p21-GFP and PCNA-RFP was detected to sites of DNA damage. Rapid relocation was also observed with DNA repair proteins, like DNA polymerase δ(pol δ), FEN-1 and CAF-1. Co-immunoprecipitation experiments with anti-GFP antibody confirmed the existence of a chromatin-bound protein complex including p21-GFP, PCNA and pol δ. Similar results were also obtained on human normal fibroblasts irradiated with UV-C light. The relocation of p21 was found to depend on the interaction with PCNA, since a p21 mutant unable to bind PCNA did not co-localize with this protein at the irradiated sites. These results suggest that p21 protein is recruited very early to DNA damage sites where it may help the turnover of PCNA-interacting proteins during DNA repair.

5

CYTOTOXIC EFFECTS AND CELL CYCLE PHASE PERTURBATIONS INDUCED BY RESVERATROL, A CHEMOPREVENTIVE AGENT, EVALUATED BY USING A TRIDIMENSIONAL ORGAN-LIKE CULTURE MODEL

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Multicellular tumor spheroids system (MCTS) is an "*organ like*" model suitable to investigate the regulation of cell differentiation and death, immune response and homeostasis. MCTSs preserve the morphological and biochemical properties of the corresponding tissue and remain in a functionally active state for many weeks, allowing to investigate the long-term exposure to various drugs. Recent studies demonstrated that resveratrol, a polyphenol present in grapes and wine, induces a drastic growth inibitory effect triggering the apoptotic process in MDAMB-231, a highly invasive and mestastatic cell line of human breast carcinoma (Scarlatti et al., FASEB J, Oct 16, 2003). In our study we approach the importance of the cell-cell interaction and the cell cycle regulation in a tridimensional organ like culture; we demonstrate that in our model resveratrol induce a drammatical cytotoxic effect (dose dependent) associated to an increase of endogenous ceramide. Flow cytometric cell cycle analysis, performed at 5 and 10 days after tridimensional culture, shows in untreated cells a significant reduction of the S/G_2M fraction with a consequent increase of the G_1 phase. Resveratrol, at the concentrations ranging from 32 to 64 μM, impairs the formation of spheroids and strongly blocks the cells in the S/G_2M phases of the cell cycle. The clonogenic potential of MDA-MB-231 cells was highly reduced by resveratrol in a dose dependent manner. This study shows that the tridimensional organ-like culture model might be a uselful and interesting approach to evaluate in vitro the antitumor activity of different compounds.

6

MORPHOLOGIC AND CYTOFLUORIMETRIC STUDY OF THE RAT COLONCARCINOMA DHDK12TRB CELL LINE AFTER BORON NEUTRON CAPTURE THERAPY

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The effectiveness of the boron neutron capture therapy (BNCT) largely depends on the neoplastic cells boron compound uptake. Despite a delivered therapeutic radiation dose, in vitro experiments, on the boron enriched rat coloncarcinoma DHDK12TRb cell line, evidenced a treatment

resistant cell fraction whose quantification and characterization are the aim of the present study. Conventional low concentration plating efficiency failed to evidence the surviving fraction that could be observed only when higher concentrations ($2x10^6$) of treated cells were reseeded in culture flasks. Exponentially growing cells incubated with 20 ppm BPA for 18 h, after neutron radiation ($7x10^{12}$ cm^{-2}, 10′), were seeded in two culture flasks for each established observation time (24 hs, 48 hs, 5, 7, 9, 15, 21 days): one designed for the cell morphology study while the other one for cell counting and flow cytofluorimetric DNA analysis. Irradiated cells lacking boron enrichment and non-irradiated cells with and without boron where similarly studied. At 24 hs cells showed no differences in all the four studied conditions, in terms of number of cells recovered in the flasks, morphology and cell cycle distribution. No boron toxicity was evidenced. Starting from 48 hs to 7 days irradiated boron enriched cells showed progressive dimension increase, cell number reduction and multiclonal DNA profile with progressively higher DNA content populations. After 9 days normally sized cell clones appeared, thus confirming the presence of a resistant cell fraction able to restore the original cell population after 21 days. The incidence of surviving cells resulted in 0,025%. Subsequent experiments will be planned to validate our preliminary results showing that cell proliferation status influences BPA uptake and therefore therapy effectiveness.

7

EFFECT OF $PbCl_2$ ON NRK-52E CELL LINE: CYTOMETRIC STUDY

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Introduction: Lead is an important environmental pollutant whose toxic effects involve the nervous system, lung, liver and kidney and it is currently listed as one of the most hazardous substances by the Agency of Toxic Substances and Disease Registry (1999). Since the proximal tubular epithelium is one of the primary target of inorganic Pb, NRK-52E, a rat kidney epithelial cell line, represents a suitable *in vitro* model for lead toxicity study. **Methods:** *Pb Treatments*. NRK-52E cells, in logarithmic growth phase, were exposed to 5, 20 μM $PbCl_2$ for 24, 48, 72 h. After each Pb treatment, cell density was determined by direct counting with hemocytometer. *Flow Cytometry.* The detection of necrotic or apoptotic cells was performed by anexin V and propidium iodide (IP) staining (Apoptest™–FITC Nexins Research). Cell cycle analysis was performed using IP staining of DNA. All the experiments were performed using a laser Epics Profile. **Results and Conclusions:** $PbCl_2$ inhibited NRK-52E cell growth rate in a dose- and time-dependent manner. This effect was maximum at 20 μM $PbCl_2$ and already evident after 48 h from the beginning of treatment. ApoptestTM–FITC evaluated that PbCl2 did not induce apoptosis or necrosis within 48 h of treatments, only after 72 h at 20 μM we observed an increase of necrotic and apoptotic cells. Cell cycle analysis showed that $PbCl_2$ increase the percentage of cells in the G_0/G_1 border already after 48 h with 5 μM, suggesting a capacity of Pb to arrest the cell cycle in G_0/G_1 phase. At 72 h, no significant differences were found between Pb-treated or untreated cultures because of the confluence. On the basis of the experiments above, $PbCl_2$ has been hypothesized to modify cell cycle regulation.

8

MODULATION OF JUNCTIONAL COMMUNICATIONS BY X RAYS AND RESVERATROL IN HUMAN GLIOMA CELLS

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Resveratrol has recently been the focus of a lot of attention due to its involvement in the modulation of several biological processes, including carcinogenesis. However, the molecular mechanisms underlying this antitumorigenic activity are still not defined. A known cellular event associated with tumour promotion is the modulation of gap junction intercellular communication (GJIC) and the ability in modulating the connections may be a useful tool for the screening and the assay of chemopreventive natural products. Gap Junctions (GJs) are collections of channels constituted by proteins encoded by the "connexins" gene family, that directly connect neighbouring cells providing the diffusion of small molecules. It has been proposed that GJs play a crucial role in growth control, so that the loss of GJs occurs during the promotion/progression steps of carcinogenic pathway, during neuropathy and teratogenesis. Transient changes in GJs have been observed during normal cell cycles, cell communications being moderate during G1/S, increased through S and decreased during G2/M. We investigated the effect of Resveratrol on GJICs in relation to its ability to modulate cell cycle progression, in a human glioma cell line. Furthermore, because the radiotherapy is the most frequently used in the management of human glial tumors, we analysed the effect of X rays alone or in combination with resveratrol, in order to verify the ability of the compound to act as a radiosensitizer. A modified two-dye cytofluorimetric assay was performed to measure GJICs in relation to cell cycle phases, and the expression of Connexin 43 and related kinases was assayed by western blotting. Our data show that both X rays and Resveratrol increase GJICs and are able to modify cell cycle progression.

9

CFSE IN THE EVALUATION OF ANTIPROLIFERATIVE ACTIVITY OF ANTICANCER DRUGS

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Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a lymphocyte tracking dye. Both migration and proliferation can be detected in cells loaded with CFSE. This ester is

metabolised by cellular esterases and forms fluorescent conjugates with proteins, which remain trapped within the cells for several days and can be detected by flow or static fluorescence cytometry. In mitosis, fluorescence is distributed evenly between siblings, which thus have half the fluorescence intensity – on average – of their mother cells. However, the use of CFSE to test the effects of antiproliferative drugs in vitro is very limited in leukemic cells, and has not been attempted, to our knowledge, with non-hematological mammalian cell lines, probably because of methodological problems. In particular, the easily-identifiable fluorescence peaks usually obtained by labeling lymphocytes are not obtained by labeling other cell types, which have a much less homogeneous protein content. We present a procedure enabling to evaluate CFSE efflux and to fix the peak positions with good approximation in advance. Histograms were fitted by a series of gaussians, each representing cells in a given generation. Then, a data-analysis procedure allowed us to quantify the time course of the percentages of cells in each generation. We are currently applying the method to evaluate antiproliferative effects of anticancer drugs in tumor cell cultures.

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ISOLATED PERFUSED LIVER IN PIGS TREATED WITH CARBON MONOXIDE AND BILIVERDIN

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Carbon monoxide (CO), one of the downstream mediators of heme oxygenase, at not-lethal concentration, mediates potent anti-inflammatory effects: vasodilatation, inhibition of platelet clotting and cell apoptosis, preventing hepatic ischemia/reperfusion (I/R) injury. AIM OF THE STUDY: 1) Improving: the function of the organ extending the pool of marginal donors and decreasing the extent of acute and chronic rejection; 2) decreasing apoptosis and other cold preservation-damages, extending the organ preservation time; 3) improving the function of the Bio Artificial Liver increasing the viability, the synthetic and metabolic capability of hepatocytes; A liver was harvested under sterile conditions according to the rapid method used in clinical liver transplantation. Duroc pigs were anesthetized and placed on mechanical ventilator inducing a level of carboxyhemoglobin not exeeding 20%. CO was administered (500 ppm) either by endotracheal tube in ventilated animals and/or via portal vein through a saturated solution (Celsior). The recipient pigs underwent a ligation of the portal vein and hepatic artery following portacaval shunt. The isolated liver was perfused through the portal vein with arterial blood from the recipient pig by an infusion pump. The blood returned to the recipient through the femoral vein. The monitoring of the function was performed until 12-h Biopsies of the liver for tissue activity studies and apoptosis was taken at the following times: just before and after the cold perfusion (18 h ischemia maximum time used in clinical transplantation); after reperfusion and every four hours before stopping the perfusion of the isolated liver. The bile production of the perfused liver was measured at 1 h intervals. Samples of the inflow blood into the liver graft and outflow blood were also taken to evalute the metabolic activity of the isolated perfused liver, especially ammonia consumption and urea production. Morphological features were recorded for each bioptic sample on H/E stained sections; the proliferation index was calculated by image-analysis evaluation of the immunostaining with anti-ki67(MIB-1) and apoptosis, evidenced by APOP-TAG, CASPA-TAG; moreover, for each case was recorded the immunoreactivity for anti-bcl-2, anti-survivin and anti HSP 27-70. **Results:** CO exerts positive effects on liver regenerative capability, particularly for that concerning the decrease of apoptosis. Biliverdin shows also a synergistic positive effect.

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THE FARNESYLTRANSFERASE INHIBITOR R115777 (*ZARNESTRA*) SYNERGIZES WITH IONIZING RADIATION IN INDUCING CELL CYCLE PERTURBATION AND GROWTH INHIBITION OF HUMAN EPIDERMOID HEAD AND NECK CANCER CELLS

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The activation of *ras*-mediated pathway is involved in tumours cells survival and in the induction of resistance to ionizing radiation (RT) in head and neck squamous cell carcinoma (HNSCC). The suppression of *ras* activation in HNSCC could enhance RT citotoxicity. In order to evaluate this hypothesis we used the farnesyltransferase inhibitor R115777 (*Zarnestra*) in combination with RT in human HNSCC "in vitro" models. Using cell growth inhibition data derived by MTT assay, we performed the isobologram analysis of the median effect developed by Chou and Talalay (*Calcusyn*, Biosoft, USA). Using R115777 and RT in a ratio of 1:2 and 1:4, we recorded a significant synergism on growth inhibition of HNSCC KB cells after 72 hrs of treatment. On the basis of data derived from *Calcusyn* analysis, we evaluated the effects of increasing doses of RT (from 1 to 6 Gy) in combination with either 0.05 or 0.1 μM of R115777 on colony formation of KB cells. Experimental data showed an increase of clonogenic growth inhibition when 2 and 4 Gy were combined with either 0.1 or 0.05 μM R115777. DNA-flow cytometry demonstrated that the treatment of KB cells with 4 Gy and 0.1 μM R115777 induced an accumulation of KB cells in S-phase (41% vs. 26.5% of control cells) while single treatment of both agents did not cause significant changes in S-phase. Moreover, flow cytometry demonstrated a rise of cell death phenomena in the same experimental conditions. These data suggest the

design of new strategies based on the combined use of RT and farnesylation inhibitors in the treatment of human HNSCC.

Supported by MIUR and AIRC

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INHIBITION OF POLY(ADP)RIBOSE POLYMERASE-1 ACTIVITY INCREASE CYTOTOXIC EFFECTS OF THE TOPOISOMERASE-I INHIBITOR TOPOTECAN ON HUMAN GLIOBLASTOMA CELL LINES

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DNA strand breaks formation activates the poly (ADPribose) polymerase-1 nuclear enzyme (PARP-1) to catalyse the transfer of the poly(ADP)ribose polymers (pADPRs) from the respiratory co-enzyme NAD+ to several nuclear proteins. The poly(ADPribosyl)ation of these proteins, at the site of DNA breakage, converts the DNA breaks into intracellular signals that activate DNA repair programs or cell death options. The direct interaction between PARP-1 and topoisomerase-I have been demonstrated and several data indicate that inhibition of PARP1 activity may enhance the cytotoxicity of camphotecins. We investigated cell growth inhibition, cell cycle perturbation and changes in gene expression in two human gliobastoma cell lines, D54 $p53_{wt}$ and U251 $p53_{mut}$, after the treatment with topotecan (TPT), in the presence or not of the PARP-1 inhibitor NU1025. In both D54 $p53_{wt}$ and U251 $p53_{mut}$ cells, MTT assay showed the synergistic effect of non cytotoxic doses of NU1025 (10 mM) on TPT-induced growth inhibition, calculated by Chou and Talalay method (*Calcusyn*, Biosoft, USA). DNA-flow cytometry showed that PARP-1 inhibitor NU1025 strongly increased the G_2M block induced by TPT treatment in both cell lines, with a longer perturbation in U251 $p53_{mut}$ cell line. Moreover, DNA-flow cytometry and PARP-1 fragmentation analyses demonstrated that combined treatment with TPT and NU1025 induced an increase of apoptosis in U251 p53mut cells. These data indicate that the inhibition of PARP-1 activity enhance the cytotoxicity of TPT in both $p53_{wt}$ and $p53_{mut}$ human glioblastoma cell lines.

Supported by MIUR and AIRC

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CELL CYCLE DISTRIBUTION OF PROTEINS INVOLVED IN PRE-REPLICATION VERSUS DNA REPLICATION AS ANALYSED BY FLOW-CYTOMETRY

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DNA replication is a highly controlled and coordinated process required for the maintenance of genome integrity. The major regulatory checkpoint in the eukaryotic cell cycle is the transition from G1 to S phase. In particular, S-phase entry is regulated by the previous assembly of the pre-replication complex (pre-RC) at origins of DNA replication. In this study we have investigated the cell cycle distribution of proteins involved in pre-replication versus replication processes. Proliferating human fibroblasts (HF) and HeLa cells were analysed by flow cytometry using antibodies against markers of pre-replication complex, MCM2, MCM7, cdc6, and against markers of replication complex, PCNA, CAF1 and DNA polymerase δ. We have studied the total amount of proteins in comparison with the chromatin-bound fraction. The results indicate that the total amount of MCM2, MCM7 and cdc6 increased from G1 to S, to G2+M phases, while the chromatin-bound forms were higher in G1 than in S phase. MCM levels reached background levels at the end of S phase, and returned to contents similar to G1, at the subsequent G2+M phases. Cdc6 showed a similar behaviour in normal fibroblasts, while in HeLa cells the protein was found in the chromatin bound form also in S phase. The proteins that participate in the formation of replication complex showed a cell cycle distribution similar to the pre-replicative proteins as far as the total amount is concerned. In contrast, high levels of the chromatin bound fraction were found exclusively in S phase. These results show that flow cytometry allows a precise temporal determination of the pre-replicative and replicative protein binding to chromatin.

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EXTRUSION OF NUCLEAR PROTEINS DURING APOPTOSIS

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During apoptosis, the regulated disruption of the chromatin compartment involves the whole nuclear structure and is paralleled by a dramatic reorganization of all the ribonucleoprotein (RNP)-containing structures which are involved in the transcription, maturation and export of RNAs (1,2). In early apoptotic cells, RNPs segregate in the interchromatin space to form heterogeneous clusters we called HERDS (for Heterogeneous Ectopic RNP-Derived Structures 3), which then move into the cytoplasm. Also several other nuclear proteins (among which PARP-1, the splicing factor SC35, fibrillarin, PCNA, c-Myc) follow a similar fate and may be recognized immunocytochemically in the cytoplasmic aggregates. These heterogeneous nucleoprotein clusters are finally extruded inside apoptotic bodies blebbing at the surface of late apoptotic cells; noteworthy, most of their protein moieties can still be here labelled by specific antibodies. The sub-cellular particles released as apoptotic bodies may be heterogeneous in size and content, and completely *new* molecular aggregates of nuclear origin are formed, resulting from partial proteolytic and nucleolytic cleavage of cytoplasmic and nuclear substrates: this is consistent with the suggestion that apoptotic cells can be a potential source and a privileged reservoir of auto-antigens, which could possibly play a role in the aetiology of autoimmune diseases.

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INHIBITION OF AURORA KINASES AS A NOVEL STRATEGY TO TREAT MALIGNANT MESOTHELIOMA (MMe): CYTOKINETIC AND PRO-APOPTOTIC EFFECTS

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Aurora Kinases are a family of serine-threonin kinases essential for centrosome duplication, chromosome segregation and cell division processes. These kinases have been shown to be over-expressed in several human cancers. We investigated the effects of an Aurora inhibitor (a 3-aminopyrazolo derivative) on cell proliferation, cytokinetics and cell viability of three MMe cell lines. A consistent inhibition of cell proliferation has been observed even at the lowest concentration tested (1 μM). A striking effect on the cell cycle profile was observed after treatment of cells as compared to controls. Cells accumulate in G2-M phase of the cell cycle and the generation of polyploid cells as detected by morphology via fluorescence microscopy was observed. An accumulation of DNA fragments in the sub-G0/G1position of the DNA histograms, directs towards an induction of apoptosis by the compound which was also supported by changes in morphology. The appearance of polyploid cells was likely due to inhibition of cytokinesis and the lack of formation of a mitotic spindle which is necessary for proper separation of chromosomes. In order to confirm the mechanism of action of the drug we evaluated its effect on the phosphorylation of histone H3 a known substrate of Aurora-B kinase, and a crucial step in the process of chromatin condensation. Our experiments show that inhibition of Aurora kinases has the potential of a new molecular target for the treatment of MMe, a tumour type still having a high unmet medical need.

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MONOPARAMETRIC AND BIPARAMETRIC CELL CYCLE RELATED ANALYSIS BY USING TO-PRO 3

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TO-PRO 3 iodide (TP3) is a monomeric cyanine nucleic acid stain, has been tested for mono-parametric and biparametric cell cycle analysis. TP3 has a peak absorbance at 642 nm and a red emission at 661 nm, and it is best excited by a HeNe laser (633 nm). TP3 has been used on peripheral blood lymphocytes (PBL) and on different cancer cell lines growing in vitro to evaluate the staining kinetics in relation to its affinity to double-stranded DNA of the different cell types. TP3 is impermeable to living cells and different methods were tested to fix the cells before DNA staining. A solution of GM saline and 96% ethanol (70% ethanol final concentration) or 1% formaldehyde (methanol-free), followed by 70% ethanol, gave the best results both in term of CV of the G_1 peak and minimal amount of debris. Different concentrations of TP3, in a range from 0.1 μM to 2 μM, were tested on the different cell lines. A linear increase in G1 peak position of the TP3 fluorescent signal up to 1 μM was found. At 2 μM the G_1 peak position decreased with a concomitant increase in the G1 peak CV. Best results were obtained with 0.5 μM TP3 in most of cell lines used. However on PBL and two leukemia cell lines 0.25 μM TP3 was enough to have good DNA histograms. Sampling at different time points were performed in all the cell lines used showing that 15 min incubation time are enough to obtain excellent staining. RNAse treatment must be included in the protocol for DNA staining. TP3 can be used also in many biparametric analysis such as DNA/BrdUrd, DNA/apoptosis, DNA/cyclin and DNA/cellular antigens. In conclusion, TP3 is an excellent alternative for propidium iodide in analyzing cell cycle by flow cytometry.

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DIFFERENT EFFECTS OF RIBOSOME BIOGENESIS INHIBITION ON CELL PROLIFERATION IN RETINOBLASTOMA PROTEIN- AND p53- DEFICIENT AND PROFICIENT HUMAN OSTEOSARCOMA CELL LINES

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There is increasing evidence that a continuous cross talk exists between ribosome biogenesis and the mechanisms controlling cell cycle progression. This reciprocal control is of great importance for continuously dividing cells in that a defective ribosome biogenesis might be responsible for the generation of ever smaller cells until the proliferation would be no longer possible. In the present study we evaluated the effects of inhibition of rRNA synthesis by low dose (0.04 μg/ml) Actinomycin D (Act D) on cell cycle progression and cell population growth in two osteosarcoma cell lines: the RB- and p53-proficient U2-OS cells and the RB- and p53-deficient SAOS cells. As far as the cell cycle progression is concerned, flowcytometry analysis demonstrated that 1 hour Act D treatment induced in the asynchronously growing, RB- and p53-proficient U2-OS cells, a block at the cell cycle check points G1-S and G2-M which was removed when rRNA synthesis was resumed. Unlike U2-OS cells, the reduced synthesis of rRNA did not influence the cell cycle progression in the

RB- and p53-deficient SAOS cells. In order to study the inhibitory effect of rRNA synthesis on cell population growth, we treated asynchronously growing U2-OS and SAOS cells with low dose Act D for 1 hour/day for 7 days. This treatment only lengthened the doubling time of the RB- and p53-proficient U2-OS without hindering the cell population growth. On the contrary, the cell population growth of the RB- and p53-deficient SAOS cells, was completely inhibited. These results demonstrate that the inhibition of rRNA synthesis causes the extinction of the cancer cell line lacking RB and p53, very likely due to a progressive reduction of ribosome content.

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APOPTOSIS OF NK CELLS FOLLOWING CYTOKINE STIMULATION

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The maintenance of immune system homeostasis is controlled by finely tuned signals of survival/proliferation and apoptosis that delete potentially autoreactive cells and close immune response. IL-2 and IL-15 are capable to activate NK cells and generate lymphokine-activated killer (LAK) cells that are more sensitive to apoptosis. LAK cell activity is controlled by different feedback regulatory mechanisms: i) the decrease of cytokine concentration that, acting on Bcl-2 family member expression, makes NK cells more sensitive to apoptosis (**1**); ii) the target cell-induced apoptosis mediated by CD18 (**2**); iii) the apoptosis induced by TNF ligand family members: **a**) Fas ligand, **b**) TNF-alfa; iv) the NK cell death upon cross-linking of CD16, CD2 and CD94. Our goal was to study apoptosis sensitivity in IL-2 or IL-15 stimulated NK cells. To this aim CD56+ LAK cells were evaluated for: 1) CD95L, TRAIL, CD95 (Fas), TRAIL-Rs and TNF-Rs surface expression; 2) Bcl-2 and TNF-alfa intracellular expression; 3) apoptosis induced by TNF-alfa administration or by cross-linking with anti-CD16, -CD18 and -CD95. Our data indicate that IL-2 or IL-15 maintain high expression of Bcl-2 and induce expression of TRAIL, CD95L, TNF-alfa and some of their receptors, TRAIL-R2, CD95, TNF-RI and -RII on NK cells. Cell death induced by anti-CD16 and -CD18 cross-linking was usually limited (5–20%); moreover LAK cells were not sensitive to TNF-alfa, TRAIL and CD95L, indicating that surviving NK cells selected during IL-2 or IL-15 activation were resistant to TNF family member mediated apoptosis.

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Environmental Sciences and Toxicology

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MULTIPARAMETRIC METHOD FOR DETERMINATION OF SPERM ABSOLUTE COUNT AND ASSESSMENT OF SPERM QUALITY

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Background: Sperm concentration, apoptosis and viability and the presence of leukocytes in semen samples can be assessed by numerous methods, but many are slow and poorly repeatable, and subjectively assess only 100 to 200 spermatozoa per ejaculate. Detection of more parameters in sperm samples may help evaluate sperm quality. The aim of this study was to develop a rapid cytometric method to determine absolute sperm count and to examine simultaneously other sperm parameters in semen samples. **Methods:** Sperm viability and apoptosis were determined on the basis of staining with Syto-16 and 7-AAD. Fluorescent microspheres were used to determine the sperm absolute count per ml. CD45 APC and CD95 PE were used to enumerate the concentration of leukocytes in semen sample and the presence of Fas in sperm cells. Fluorescence staining was performed in a single test tube containing 100 µl of semen sample and flow cytometric analysis was performed using a dual laser FacsCalibur four-colour with CellQuest Software, acquiring 100,000 events for sample. **Results:** Cytometric data collected from 50 samples were compared with results from microscopy examination and morphological test routinely used to evaluate sperm quality. We found a good correlation in absolute count of spermatozoa ($r = 0.84$).But if we don't consider the very high values for which manual count presents objective difficulty the correlation was better $r = 0.98$. The variation of replicate measurements within 5 replicate samples was low ($SD = 2.09$). CONCLUSIONS: This cytometric assay is found to be rapid sensitive and reproducible measure of semen quality.

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INACTIVATION OF FECAL INDICATORS AND BACTERIA IN ACTIVATED SLUDGE BY ULTRASOUNDS

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The application of ultrasounds at 20 kHz frequency causes the progressive damage and death of viable microorganisms, depending on the applied energy level, as a function of time of treatment and applied power. The importance of knowing the effects on bacteria integrity is relevant for the optimisation of sonication in the field of wastewater treatment or disinfection. For example, a sequential inactivation of bacteria combining sonication with UV irradiation, ozone

or hydrogen peroxide dosage can be applied to enhance water or wastewater disinfection. In this research the effects of ultrasounds on viability of microorganisms present in activated sludge taken from a municipal wastewater treatment plant, as well as on fecal indicator bacteria such as fecal coliforms (*E. coli*) and streptococci (*Enterococcus faecalis*) were investigated. Different levels of ultrasounds were applied, at *specific energy* (E_s) up to 300 kJ L^{-1}. Flow cytometry was used for the identification and quantification of viable and permeated bacteria, after the fluorescent staining of cellular nucleic acids with Sybr-Green I and Propidium Iodide. The main results showed that: (i) *E. coli* was high sensitive to the application of ultrasounds, showing a reduction of 76% of viable cells at E_s of 220 kJ L^{-1}; (ii) on contrary *Enterococcus f.* was more resistant than E. coli and a viable cells reduction of 16.5% was observed at 220 kJ L^{-1}; (iii) bacteria in activated sludge exhibited a dynamic of viable and permeated bacteria similar to *Enterococcus f.*, indicating a high resistance to physical-mechanical stress, such as ultrasonic cavitation. Summing up, in the investigation on activated sludge it was observed that low levels of E_s causes a prevalent disaggregation of flocs releasing single cells in the bulk liquid, while a net loss of bacteria due to their disruption was induced only by very high levels of energy (over 160 kJ L^{-1}).

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DETECTION OF FLUORESCENT LIPOSOMES IN THE COPEPOD GUT USING CONFOCAL LASER SCANNING MICROSCOPE

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Liposomes are small particles composed of a lipidic bilayer surrounding one or more aqueous cavities, largely used in the pharmaceutical and cosmetic fields. Liposomes are biodegradable and not toxic and the presence of hydrophobic and hydrophilic portions makes them a very versatile carrier allowing for the encapsulation of both lipophilic and hydrophilic compounds. In this study we used giant liposomes as a delivery system of different bioactive molecules to study their effects on the reproductive physiology of copepods. In aquaculture, small liposomes have already been used as nutrient supplements or as drug carriers in first-feeding marine fish larvae, but they have never been used as a delivery system for copepods. Copepods are small crustaceans at the base of the marine food web. Since they represent the principal diet for many fish larvae, fluctuations in copepod biomass can dramatically influence fish species abundances at higher trophic levels. Copepod egg production rates and egg-hatching success are key biological parameters to predict secondary production at sea, both of which are strongly influenced by quantity of the available food or by quality, such as the presence of toxicants in the food, that negatively impact copepod reproductive fitness. In our study, liposomes have been prepared with a mean size comprised in the same range of food ingested by copepods (mean diameter of about 7 μm). The encapsulation of an hydrophilic and high molecular weight fluorescent compound, fluorescein isothiocyanate-dextran (FitcDx), within the liposomes, allowed to verify copepod ingestion and palatability using the confocal laser scanning microscope. Females of the calanoid copepod *Temora stylifera* were fed with FitcDx-encapsulated liposomes alone or mixed with the dinoflagellate alga *Prorocentrum minimum*. Control copepods were incubated with the P. minimum diet alone. Egg production rates, percentage egg-hatching success and number of fecal pellets produced were evaluated after 24 and 48 h of feeding. Epi-fluorescence of copepod gut and fecal pellets indicated that the liposomes were actively ingested by *T. stylifera* in both experimental food conditions, with or without the dinoflagellate diet. On the contrary, egg production and hatching success were very low with a diet of liposomes alone and fecal pellet production was similar to that recorded in starved females. This suggests that liposomes alone did not add any nutritive value to the diet, making them a good candidate as inert carriers to study copepod physiology in ecotoxicological experiments.

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VIABILITY ASSESSMENT IN PHYTOPLANKTON BY FLOW CYTOMETRY: ECOTOXYCOLOGICAL APPLICATIONS

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Flow cytometry represents a very valuable tool to assess physiological responses of unicellular algae to environmental stresses at the single cell level. Diatoms are Chromophyte algae very successful in coastal systems, representing 40% of total marine primary production. Using inherent optical properties, such as scattering, or fluorescent stains, we have investigated stress responses of diatoms to unsaturated aldehydes, which are produced by several species of diatoms as a chemical defense against their predators. Such compounds have been shown to affect diatom cell membrane integrity, to interfere with the progression of their cell cycle, to induce NO production and finally lead to death by a mechanism similar to apoptosis of mammalian cells. Flow cytometry has been proven to be invaluable in detecting and estimating the intrapopulation variability in the response to the toxicant, showing the generation of several subpopulations upon exposure to the aldehyde. Preliminary characterization of these subpopulations suggests that dividing cells are most affected by aldehydes, probably due to the demonstrated effect of these compounds on tubulin organization. This study has strong ecological implications, as evidence is accumulating that aldehydes are released during diatom blooms due to cell lysis. It is reasonable to expect that

they are able to affect other diatoms as well as other phytoplankton species. However, their role should not be limited to toxicity only, as they might act as signals to individuals of the same population, and may represent effectors of the final stages of a bloom at sea.

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USE OF EXFOLIATED BUCCAL CELLS TO EVALUATE EARLY GENOTOXIC EFFECTS OF OCCUPATIONAL EXPOSURE TO ASPHALT FUME IN PAVING WORKERS

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Paving workers are chronically exposed to polycyclic aromatic hydrocarbons (PAHs) contained in asphalt fumes. We evaluated early genotoxic effects in 16 paving workers and 16 controls. Environmental and biological monitoring of exposure was carried out measuring, on personal air samples from exposed workers collected during 3 working days, 14 PAHs and urinary OH-pyrene. Micronucleus analysis and Fpg-modified Comet test on exfoliated buccal cells were used to evaluate early genotoxic and oxidative effects. Oxidative DNA damage was assessed calculating tail moments from fpg-enzyme treated cells (TMenz) and DNA damage was also evaluated analysing comet percentage. Personal air sampling showed a low level of mean total PAHs (3,17 $\mu g/m^3$) with prevalence of 2–3 ring PAHs (3,03 $\mu g/m^3$). Urinary OH-pyrene after three days were significantly higher than that found at the beginning of working week (0.76 vs 0.24 μg/g creatinine). Exposed group showed a slight higher micronuclei frequency than controls (0.42‰ vs 0.39‰). Fpg Comet test evidenced in exposed, a slight increase of mean TMenz value as compared to controls (44 vs 39). Also comet percentage was in exposed higher than in controls (21% vs 12%) ($p = 0.058$). The results show the induction of slight genotoxic and oxidative effects by asphalt fumes that correlate with the low PAH exposure level. Moreover they show that the application of both the techniques on exfoliated buccal cells, obtained by noninvasive procedure, could represents a biomarker of occupational exposure to complex mixture of chemicals at low doses.

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CYTOMETRY OF SUPRAMEDULLARY NEURONS IN SOLEA OCELLATA

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The supramedullary neurons (SN) are a noradrenergic autonomic system of giant cells, dorsally grouped in a cluster at the boundary between medulla oblongata and spinal cord in some orders of fish, and aligned one by one along the spinal cord in others. Morphology, number and size are peculiar for each species. The axons of SN terminate in the epidermal layer near the mucous glands. Because of the role of aspecific defence of skin mucous cells in fish, the SN system may acts in protection from predaction or prevent infection. In this view the study of SNS is very interesting in species fishing of considerable market price. The SN of Solea ocellata, about 70, are distributed in small groups of 2–4 along the cord. The unique nucleolus is intensely basophilic. Vacuoles and endocellular capillaries are present in the cytoplasm, as in SN clustered. The cytometry showed a large variability and a wide range of cells and nuclei size. On the contrary, the nucleoli showed an almost uniform size. The nuclei showed an intense Feulgen-positivity. The cytofluorimetric evaluation revealed a DNA content ranging from 6C in the smaller neurons to 100C in the larger ones, indicating that the peculiar phenomenon of DNA amplification is present also in the SN of S. ocellata, as so far known in those clustered only. Thus, the SN of S. ocellata have characteristics similar to those clustered and those aligned. The case of S. ocellata suggests that the parting of SN in "aligned" and "clustered" should not be interpreted in absolute way, because this neuronal system shows in different species a wide morphological variability, which reflects functional differences, probably related to environment and behaviour.

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FLOW CYTOMETRIC APPLICATION TO THE STUDY OF MORPHOLOGICAL AND PHYSIOLOGICAL CHANGES IN *VIBRIO PARAHAEMOLYTICUS* DURING SURVIVAL IN THE MARINE ENVIRONMENT

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The genus *Vibrio* includes a different group of heterotrophic marine bacteria including many pathogenic strains. In response to their environmental conditions, bacteria may be present in a viable but non culturable state (VBNC), and still be responsible for cases of human infectious diseases. In this study a *V. parahaemolyticus* strain, maintained in a specific seawater microcosm at 4°C until the acquisition of VBNC state, was periodically monitored to study: i) total cell count using an indirect immunofluorescence technique and acid nucleic staining in Flow Cytometry (FCM) and epifluorescence analysis; ii) bacterial respiration using the CTC physiological probe; iii) membrane integrity in FCM by the double DNA staining NADS protocol (SybrGreen I and Propidium Iodide applied simultaneously); iv) the morphological changes by FCM and electron microscopy; v) changes in virulence expression (haemoagglutination and hemolysin production). The NADS staining evidenced the presence of a live

population during the non-culturable state where no changes were observed on virulence expression. Moreover, we investigated bacteria resuscitation from VBNC state (after the 69th day of culture) in different culture conditions. Goal was achieved by altering temperature. Finally, FCM happened to be a sensible method for detect end evaluate the viable but non culturable pathogenic bacteria state. Supported with a CIPE grant n.17/2003, Marche region.

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TESTING THE FLOW CYTOMETRIC DOUBLE STAINING PROTOCOL IN REALISTIC PLANKTONIC BACTERIAL DEATH SITUATIONS

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Since bacteria play an important biogeochemical role in aquatic ecosystems, and have a high capacity to survive in extreme environments, researchers are searching for protocols that might give hints on the effect of environmental variables on their physiological state. We tested the double DNA-staining method (NADS, Gregori *et al.* 2001) with conditions that are likely to generate bacterial death. Seawater was collected in the coastal Mediterranean area to prepare four microcosms: two with unfiltered seawater (diluted and not diluted with <0.2 μm water) and two with water filtered through 0.8 μm filter to eliminate flagellates predators. The effect of heat, ultraviolet treatments (UVC), and an antibiotic cocktail on heterotrophic bacteria was tested and combined with the experimental elimination of predators. Viability was also tested with the detection of actively respiring cells (CTC+) and the uptake of tritiated leucine during 7 days of growth. In the absence of predation pressure on bacteria, we observed bacterial growth and an increase in activity and viability. Significant differences were observed between the activity during the various treatments with the death-causing treatments. The NADS protocol appears to be easy to perform and relatively high information-containing to discriminate live and dead cells in natural plankton samples. During the various death-causing treatments the NADS protocol showed a reasonable response, and similar to the detection of actively respiring cells (CTC+) and the uptake of tritiated leucine. Heat treatment induced more damage to bacteria membrane cells than UVC and the antibiotic cocktail. Predators, however, induced bacterial mortality that was well collected by the NADS method, more than the changes in leucine uptake or CTC+ cell abundance, which are changes that could correspond to a decrease in activity and not to an increase in mortality. The NADS protocol, in combination with flow cytometry appears to be an easy and unambiguous method providing relatively high information-content useful to discriminate live and dead cells in natural plankton samples. Supported with a CIPE grant n.17/2003, Marche region.

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ASSESSMENT OF SWINE SPERM QUALITY AFTER *IN VITRO* MYCOTOXIN ZEARALENONE AND ITS DERIVATIVES EXPOSURE

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Xenoestrogens (natural and synthetic products) can be present in foods and could induce modifications of reproductive functions in human and animals. Zearalenone (ZEA) and its derivatives (α and β-zearalenol) are fungal metabolites produced by Fusarium species frequent contaminants in cereals. For their known estrogenic activity in farm animals, we investigated the swine semen quality after in vitro exposure to ZEA and its derivatives (α and βZEA) by the assessment of viability (Trypan blue dye exclusion), motility (CASA), stability of chromatin structure (SCSA) and DNA fragmentation (Apo-BrdU). After 24 h, all mycotoxins, at concentrations ranging from 10^{-6} to 10^{-4} μM, modified the semen quality with different effects. ZEA induced DNA double-strand breaks (54%; $p < 0.05$), evaluated by flow cytometry, α-ZEA modified the sperm chromatin structure (64%; $p < 0.001$), and β-ZEA provoked modification of kinetic parameters. Influences on viability, evaluated by membrane damage, were induced by higher concentrations (10^{-2} to 1 μM) of mycotoxins. After 48 h of incubation, β-ZEA showed apoptosis induction (more than 50%) and increased instability of chromatin (14%) at low levels (10^{-8}–10^{-6} μM). This is the first report on toxicity induced by ZEA and its derivatives in swine spermatozoa. Exposure to ZEA and its derivatives could influence sperm quality, considering the widespread occurrence of mycotoxins in cereals and the low concentrations found to be active in this study.

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REPAIR CAPABILITY OF IRRADIATED TESTIS CELLS OF PARP-1−/− MICE

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Given the presence of continuous endogenous and exogenous damaging agents, mammalian cells evolved complex systems of protection, detoxification and repair. One of the immediate eukaryotic cellular responses to DNA breakage is the covalent post-translational modification of nuclear protein with poly(ADP-ribose) from NAD+ as precursor, mostly catalysed by Poly(ADP-ribose)polymerase1 (PARP-1). PARP-1 is a highly conserved nuclear protein that plays a role in DNA repair, recombination, proliferation and genomic stability. Chemically induced or genetic PARP-1 deficiency reduces cell proliferation, causes a high degree of chromosome aberrations and sister chromatid exchanges and increases cell sensitivity to genotoxic agents. PARP-1 is constitutively expressed

at a level depending on the type of tissue or cell. In particular, germ cells are characterized by a high expression level of PARP and PARP −/− mice, although fertile, show an increased genomic instability. This study aims to evaluate differences in spontaneous and X-ray induced DNA lesions in testicular cells from PARP−/− and wild-type mice. Furthermore, the DNA repair capability was also addressed. Testis cells were irradiated *in vivo* or *in vitro* with 4 Gy X-ray and the level of DNA damage was assessed by alkaline comet assay. In addition, the ability of germ cells to recover was investigated by analyzing cells at different times after irradiation (*in vitro*: 0, 30, 120 minutes; *in vivo*: 120 minutes and 24 hours). Results showed a similar level of DNA damage in unirradiated testicular cells from PARP−/− and control mice. 4Gy X-ray induced a comparable increase of DNA strand breaks in both groups of mice. Both in vivo and *in vitro* results on DNA repair capability suggested a delayed recovery in PARP−/− cells as shown by the higher level of residual damage in these cells, especially at short times after irradiation. In conclusion, these preliminary data support the hypothesis of a role of PARP-1 in DNA repair of male germ cells. (Partially supported by EU Contract FIGH-CT-200200210)

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RADIATION EFFECTS IN CULTURED TUMOR CELLS: PROLIFERATIVE ARREST AND APOPTOSIS

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Some tumour cells readily undergo apoptosis when exposed to ionizing radiation while others die by different pathways. In the present work two different cell lines, MCF-7 from mammary carcinoma and HeLa from cervix cancer, were studied after gamma irradiation with a single acute dose. Irradiation of HeLa and MCF-7 cells up to 20 Gy resulted in a time dependent decrease of cell viability as assessed by the trypan blue exclusion test. The effect was much stronger in HeLa than in MCF-7, causing the death of about 80% HeLa cells 4 days after irradiation whereas the same treatment resulted in about 35% MCF-7 killing. A significant increase in the percentage of apoptotic cells ($P < 0.001$) – detected as percentage of annexin-V positive cells – (33.2 ± 6.1%, 26.0 ± 5.8% and 28.0 ± 5.7% at 24, 48 and 72 h after irradiation, respectively), with respect to control cells, was found in HeLa cells. On the contrary, MCF -7cells did not show a significant increase in apoptosis with respect to not-irradiated cells throughout. Treatment of MCF-7 cells with 100 mM of BSO over an 18 h period resulted in an increase of cell radiosensitivity. At day 2, the percentage of MCF-7 killed cells are comparable to the value of killed HeLa cells. Treatment of MCF7 cells with BSO resulted in an increase of apoptosis 24 h after irradiation with a single dose of 20 Gy, the value of the percentage of annexin-V positive cells raising to 36 ± 5.7%. Cell cycle, quantified by means of FACS analysis, revealed a similar G2 arrest for both cell lines. In HeLa cells, a considerable amount of DNA fragmentation in a sub G1 peak is visible, while the same effect is not observed in MCF-7 cells. This result points to the presence of apoptosis in HeLa cells as observed by annexin-V test.

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COMET-FISH TO DETECT RADIATION INDUCED SITE-SPECIFIC DNA DAMAGE

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Ionizing radiation targets DNA molecules and produces a spectrum of lesions, including single-and double-strand breaks and alkali-labile sites. Comet-assay (single cell gel electrophoresis (SCGE)) has been widely used over the last years to study radiation-induced DNA damage and repair. A recent technique called Comet-FISH combines the single-cell assessment of DNA damage of Comet-assay with the chromosomal specificity of fluorescence in situ hybridization (FISH). The use of fluorescent-labeled DNA probes permits the localization of the region of interest within the head or the tail of a Comet offering the opportunity to follow site-specific DNA breaks within an individual cell. Here, we applied Comet-FISH in two cell lines with different radiosensitivity, RT112 (human bladder carcinoma) and SW48 (human colon adenocarcinoma), exposed to increasing doses of X-rays (2, 4, 6 Gy) to measure the level of DNA damage in p53 gene region using specific DNA probe. In addition we examined radiation-induced overall DNA damage and repair capability of these cells. Unirradiated (control) and irradiated cells of RT112 and SW48 were analyzed and number and location of hybridization signals within Comet were recorded. Control cells displayed any DNA damage as evidenced by the absence of a comet tail with two hybridization signals in intact head. After exposure to X-rays, in both cell types, the mean number of signals per cell (head and tail) increased as the radiation dose increased as well as the frequency of cells showing p53 hybridization signals localized in the tail, particularly at higher dose. These data suggest that p53 gene appear to be located in a region of the human genome sensitive to ionizing radiation.

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DIVIDE ET IMPERA: A FLOW CYTOGENETIC APPROACH TO DEAL WITH THE 17 BILLIONS NUCLEOTIDES GENOME OF WHEAT

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Bread and pasta wheat provide together over 60% of the calories for our daily life, making these species the world lea-

ders among major agricultural crops. Their genome size approach 17 and 12 billions of nucleotides, respectively, thus surpassing more than one hundred-fold the genome and more than ten times the rice genome and 5 times that of the human genome. Both wheat species are allopolyploids consisting of two and three groups of homoeologous chromosomes belonging to the A, B and D genomes (bread wheat: 2n = 6 X = 42; pasta wheat: 2n = 4 X = 28). This resulting in a large genome redundancy which poses great challenges for gene discovery and sequencing. Even at reducing costs, the amount of founds required for a whole genome sequencing would exceed several hundreds of millions of Euros. A flow cytogenetic approach allows to dissect the wheat genome into single chromosomes and chromosome arms and may represent a promising approach that could greatly simplify wheat genome analysis. A large number of viable aneuploid lines were developed for all chromosomes including a series of chromosome-arm aneuploids: monotelosomics, ditelosomics, tritelosomics and iso-chromosomics (Sears and Sears 1978), thus allowing the chromosome discrimination and identification on the basis of different DNA content. Flow Cytogenetics offers a unique opportunity for the production of chromosome- and chromosome arm-specific large inserts DNA libraries which allow the assembly of the global physical map in preparation for the sequencing of the gene containing regions of homoeologous groups of chromosome. Combining this approach with a Clone-by-Clone sequencing will provide a wide-ranging information about structure and function of a genome. Sorted chromosomes find several other applications in cytogenetics like detection of polymorphisms, gene physical mapping and high-resolution FISH localization. All these flow cytogenetic techniques first developed for animal cells, are now available for plant species, making feasible the project of investigating such large genomes.

An International Consortium (WGSC – http://www.wheatgenome.org/index.html) is now planning to start the challenging endeavour of having the complete genome sequence of bread wheat to accelerate improvement of this important crop, which will foster increased yield, a better economic development and global food security.

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COMPARISON OF DETACHMENT PROCEDURES FOR DIRECT COUNTS BY FLOW CYTOMETRY AND TO PERFORM FISH OF ACTIVATED SLUDGE BACTERIA

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The impact of wastewater microbial treatment has an enormous commercial and environmental importance. Bacteria present in the biomass are the responsible of the degradation process in a plant and until now the bacterial community and their associated metabolic activities are poorly understood. The use of flow cytometry coupled with fluorescent dyes can be a valid method to rapidly assess the number and the physiological state of activated sludge bacteria. This method can also allow to estimate the whole bacterial population, including its not cultivable fraction. The cytometric analysis requires an homogeneous cell suspension and so the detachment of bacteria from flocks is required. For this reason, the first step of this study was to find the most adequate pre-treatment method for bacterial cytometric analysis in activated sludge sample. For this purpose, different instruments and techniques were compared. Each method showed a good efficiency in term of bacterial detachment; the final choice of the best treatment was based on viability results obtained with Nucleic Acid Double-Staining protocol (NADS protocol, Barbesti et al. 2000) and on analysis rapidity. In a second step we applied Fluorescent In Situ Hybridization (FISH), which represents a useful approach to assess bacterial composition in activated sludge samples. In this study nitrifying microbial community was qualitative analyzed by FISH in samples of sludge taken from the oxidation tank, previously treated with different methods to partially disgregate flocks and cells were observed at the microscope. A quantitative analysis with an epifluorescence microscope is developing in our lab to better understand the functioning of the microbial communities in a activated sludge process. Supported with a CIPE grant n.36/2002, Marche region.

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EFFECT OF RARE EARTH ELEMENTS ON THE GROWTH OF *AGROBACTERIUM* SPP. AND *RHIZOBIUM LEGUMINOSARUM* FRANK

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Rare earth elements (REEs) enriched fertilizers are currently used in China for soil and foliar treatments to crops and accumulation in soil can take place following repeated applications. Although REEs naturally occur in the environment and in biological systems, little is known about the effect of such elements on the growth of soil borne microorganisms. The growth of *Agrobacterium tumefaciens* (Smith and Townsend) Conn. strains C58 and B49C, *Agrobacterium radiobacter* (Beijerinck and van Delden) Conn. strain K84 and *Rhizobium leguminosarum* Frank in the presence of increasing levels of REEs in the culture medium (MGY) was investigated by flow cytometry. An *in vitro* assay miniaturized procedure was performed to monitor the effect of different concentrations of either a mix of REEs (La, Ce, Pr, Nd) nitrates or lanthanum nitrate in comparison with MGY alone as control. Bacteria were grown at 28°C at 1050 rpm on an Eppendorf thermomixer and in Eppendorf Lidbac tubes. Tested concentrations of lanthanum nitrate and REEs nitrate mix ranged from 0,001 to 100 mM. The effects on bacterial populations, following different incubation times and concentration treatments, were evaluated using Fluorescein di-Acetate and Propidium Iodide and Sybr Green and Propidium iodide to assess metabolic activity and viability respectively.

Preliminary results of the effect of REEs on tested soil borne microorganisms are reported and possible effects of REEs accumulation on beneficial and detrimental soil borne populations are discussed.

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EUPLOTIN C MODULATES DIHYDROPYRIDINESENSIBLE CALCIUM CHANNELS IN *PARAMECIUM*

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When in Paramecium internal Ca^{2+} concentration, $[Ca^{2+}]_i$ coupled to membrane depolarization, increases a ciliary beating reversal, consequently a reversion of swimming direction occurs. The ciliary reversal (CR) duration is correlated to Ca^{2+} influx amount and addition of Ca^{2+} current blockers reduces the depolarization-induced increase in $[Ca^{2+}]_i$ and the duration of backward swimming. In this study the effect of euplotin C – a lipophilic toxic metabolite produced by the protist ciliate *Euplotes crassus* – on internal calcium regulation, visualized by changes in CR duration, is studied in *P. primaurelia*. Evidence is given that CR duration, induced by high external KCl concentrations, is longer in euplotin C-treated cells than in control cells. To test the hypothesis that euplotin C increases CR duration by modulating a specific subtype of Ca^{2+} channel, selective Ca^{2+} channel blockers are used. Blocking L-type channels by nimodipine and verapamil, N- and Q-type channels by ω-conotoxin, fractions GVIA and MVIIC, P-type by ω-agatoxin IVA significantly reduces the CR duration evoked by membrane depolarization, suggesting an involvement of these channels in ciliary reversal. The effect of euplotin C on CR duration persists when ω-conotoxin GVIA, ω-conotoxin MVIIC or ω-agatoxin IVA are applied and disappears when L-type channel blockers are used. The magnitude of the block by nimodipine and verapamil in the presence of euplotin C is comparable to that observed in the absence of euplotin C, suggesting that the Ca^{2+} channels modulated by euplotin C are dihydropyridine-sensible calcium channels. The presence of L-, N-, P/Q and R-type Ca^{2+}-like channels dispersed on ciliary and plasma membrane is evidenced by immunofluorescence and confocal microscopy.

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EFFECTS OF COMMON DIATOM-DERIVATED ALDEHYDES ON THE GROWTH OF SIX MARINE PHYTOPLANKTON SPECIES

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Phytoplankton species have evolved different strategies to enhance their defense capacities in biotic interactions such as grazing, viral infection and competition for resources. They may co-exist and compete by directly interfering with each other, through e.g., the release of chemical compounds. Diatoms are the most important group of eukaryotic phytoplankton in the ocean, representing 40% of marine primary productivity. Reports have demonstrated the existence of a chemical defense system where release of diatom-derived aldehydes induces abnormal development of copepods and other invertebrates, predators of diatoms. A recent study shows that the diatom derived aldehyde, 2-trans-4-trans decadienal, can trigger cell cycle arrest and induce an active cell death mechanism in other diatoms which closely resembles to apoptosis. We investigated by flow cytometry the effect of three unsaturated aldehydes produced by the diatom Skeletonema costatum on the growth rate and optical parameters of 6 ecologically relevant phytoplankton species. The results show that 2-trans-4-trans decadienal, 2-trans-4-trans octadienal and 2-trans-4-trans heptadienal induce a reduction in growth rate and the effect is stronger for decadienal compared to octadienal and heptadienal. Furthermore, all three aldehydes generate an alteration of intrinsic optical parameters in all phytoplankton species tested. The effect on FALS may be due to the observed effect of aldehydes on cell division, while RALS increase may be due to the induction of apoptosis. There is a gradient of the EC50 values depending upon the species as well as the aldehyde used. The different reactions of the tested species may reflect differences in their ability to compete with diatoms. Consequently, the production of reactive aldehydes by diatoms may further the understanding of succession of phytoplankton communities in the marine environment.

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FLOW CYTOMETRIC DNA PLOIDY IN CANINE TUMOURS COMPARED TO HUMAN: PRELIMINARY DATA

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Some pets' tumours can be useful models in comparative oncology, being strictly related to the human counterpart. Malignant melanoma (MM), squamous cell carcinoma (SCC) and non-Hodgkin's lymphoma (NHL) are frequent both in humans and in dogs. Analysis of DNA content is an important adjunctive prognostic tool in some human neoplasms, whereas it's not yet routinely performed in veterinary medicine. To test the suitability of the method in different canine tumours, to evaluate the incidence of DNA aneuploidy and to compare results with the human field, 20 MMs, 9 SCCs and 42 canine NHLs, PI stained (DNACon3, Consul TS), were subjected to flow-cytometric DNA analysis. No differences between fresh and frozen samples and between fine-needle aspiration, manual and mechanical disaggregation were detected. 20% of MMs and 2,4% of NHLs were not analyzed because of high background or CV > 8, all SCCs showed a CV < 8. The quality of histograms seemed to be linked to tissue type more than to disaggregation method. Aneuploidy rate was 18,8% in MMs (1 hypo- and 2 hyper-diploid out of 16), 33,3% in SCCs (3 hyperdiploid out of 9) and 12,2% in NHLs (5 hyperdiploid out of 41). The 3 aneuploid MMs were near-

diploid, in contrast with the human counterpart, frequently tetraploid. Lacking benign melanomas in our study, we could not verify whether aneuploidy is related to malignancy, as in humans. Among SCCs, the 3 aneuploid samples were oral (out of 4) and the only case with metastasis was one of them. These data are comparable to human oral SCCs: high rate of aneuploidy (about 50%) and higher tendency to metastasis of aneuploid SCCs. The rate of DNA aneuploidy among NHLs was similar to human NHLs; no correlation between ploidy and histomorphology, immunophenotype and prognosis was found and in human medicine conflicting data are reported.

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RELATIONSHIP BETWEEN SPERM CHROMATIN INTEGRITY AND DIETARY EXPOSURE TO PCBs AND DDT IN EUROPEAN AND INUIT POPULATIONS

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Persistent organochlorine pollutants (POPs), such as polychlorinated biphenyls (PCBs) and the insecticide dichlorodiphenyl trichloroethane (DDT), are stable lipophilic compounds widely found in the environment and organisms. POPs bioaccumulate and magnify by up to thousand times the background levels especially in top rank predators of the aquatic food chain, man included. Human exposure to POPs is ubiquitous but a high intake of fish and sea mammal food in the Arctic regions is associated with extraordinary high exposures, which exceeds recommended thresholds. Wildlife reports and laboratory studies demonstrated that PCBs and DDT can interfere with hormone homeostasis and are classified as endocrine disruptors. Whether these chemicals can also cause endocrine-related diseases in humans and impair male fertility is an issue of public concern and scientific debate. To explore the hypothesis that environmental exposure to POPs is associated with altered sperm chromatin structure integrity (known to impact the male fertility potential), a cross-sectional study including 707 adult males (193 Inuits from Greenland, 178 Swedish fishermen, 141 men from Poland, and 195 men from Ukraine) was carried out in 2001–2004. Serum levels of PCB-153, a proxy of the total PCB burden, and of p,p′-DDE, the major metabolite of DDT, were determined from individual blood samples. The flow cytometric sperm chromatin structure assay (SCSA) was used to assess chromatin defects. An increased risk of having a higher fraction of sperm with DNA breaks resulted associated with increasing PCB-153 serum level for European, but not for Inuit men, in spite of the higher POPs levels found in the latter cohort. No significant associations were found between SCSA parameters and p,p′DDE serum concentrations. Our data, obtained from the largest application of the SCSA in molecular epidemiology surveys, suggest that human dietary PCBs might affect sperm chromatin integrity and experimental strategies have been deployed to address the different regional susceptibilities emerged in the study.

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SEMINAL APOPTOSIS AND SERUM LEVELS OF CB153 AND DDE IN EUROPEAN AND INUIT POPULATIONS

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The persistent organochlorine pollutants (POPs) are stable lipophilic compounds, known to interfere with hormone activity and with the normal homeostasis of spermatogenesis. The aim of this study was to verify if germ cell apoptotic mechanisms, operating during spermatogenesis, could be influenced by the presence of POPs in the blood. This study includes 627 adult males (193 Inuits from Greenland, 160 from Sweden, 132 from Poland, and 142 from Ukraine) that have been investigated for the presence in the serum of CB-153, as a proxy of the total PCBs burden, and of p,p′-DDE, as a proxy of the total DDT contamination. Two apoptotic markers (Fas and BclxL) and DNA fragmentation (by TUNEL assay) of the subjects' spermatozoa have been evaluated by means of cytofluorimetric analyses to assess the male reproductive status. We found geographical differences in the four populations studied but, despite great contrasts of exposure to CB-153 and p,p′-DDE, no differences in apoptotic sperm parameters and DNA breaks could be related to serum levels of POPs. Surprisingly, the lowest levels of Tunel positivity were found in Inuits', who was also the population where highest exposure levels and highest exposure contrasts were obtained. Only further analysis should demonstrate whether Inuits posses a peculiar genetic background and/or are subjected to environmental conditions (for example a high dietary consumption of antioxidant molecules and/or temperature particularly favourable for a correct spermatogenesis) which provides higher sperm quality output than Caucasian men.

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ABSENCE OF GENOTOXIC EFFECTS OF RADIOFREQUENCY GSM/BASIC 935 AND 1800 MHz ON HUMAN BLOOD CELLS

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In view of the widespread use of mobile phones, concern has been expressed about the possibility of adverse health effects related to mobile telephony. Despite the gen-

eral conviction that microwaves are not sufficiently energetic to be able to directly damage DNA, the so far published research has not been able to give a conclusive answer to the question of whether radiofrequency exposure alone can affect the DNA or influence the genotoxicity of other physical or chemical mutagens. The aim of this study was to evaluate the potential genotoxic effect of radiofrequency (RF) alone or in combination with X-rays (1 Gy) on human blood cells. Three different conventional and molecular cytogenetic tests: chromosome aberrations (CA), micronuclei (MN) and alkaline comet assay were applied. Heparinized whole blood taken with informed consent from healthy non-smoker donors (10 for 935 MHz,1 W/Kg and 5 for 1800 MHz, 1W/Kg) was exposed to RF for 24 h either before or after X-rays together with concurrent appropriate sham exposures. RF exposures were done at 37°C in waveguides installed in tissue culture incubators. Immediately after treatment, blood aliquots were collected to be processed by the comet assay. Slides were analysed by a computerized image analysis system and to evaluate the amount of DNA damage, computer generated tail moment (tail length * fraction of total DNA in the tail), tail DNA fraction or tail length were used. Chromosome aberration and micronucleus assays were performed using whole blood cultures for 48 or 72 h respectively as suggested by standard protocols (IAEA, 2001). Proliferation index was also evaluated. Results did not show any significant difference between radiofrequency exposed and sham samples for each cytogenetic endpoint analyzed. Similarly, the combined exposure failed to indicate the presence of any synergistic effect between radiofrequency and X-rays. Proliferation indices did not indicate any differences between samples. This Project (PERFORM B) is partially supported by Elettra 2000, MMF and GSM association, and National Program supported by Ministry of Education, University and Research: "Human and Environment Protection from EM Emissions."

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CHROMOSOME ABERRATIONS, MICRONUCLEI AND DNA PRIMARY DAMAGE IN NURSES OCCUPATIONALLY EXPOSED TO ANTINEOPLASTIC AGENTS. INFLUENCE OF GENETIC POLYMORPHISMS

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The widespread use of chemotherapy raises concern about the high health risks for care personnel. In fact they are handling large amounts of these compounds, which can be adsorbed despite appropriate protective measures being taken. We investigated whether occupational exposure to antineoplastic agents resulted in genetic damage, possibly indicative of adverse health effects in the long term. A cytogenetic investigation (chromosomal aberrations (CA), micronuclei (MN) and DNA primary damage (Comet assay) was carried on a group of 83 workers of oncology units occupationally exposed to antineoplastic drugs and on 96 subjects of a control group (from healthy blood donors) matched for gender and age. All subjects (mean age = 36 years) were asked to fill in the personal healthy questionnaire proposed by the International Commission Protection against Environmental Mutagens and Carcinogens. Workers were also selected using a questionnaire concerning the individual occupational exposure to antineoplastic drugs. Furthermore, as specific polymorphisms in the metabolic or DNA repair genes can modulate the individual response to mutagens and carcinogens, we studied four gene polymorphisms (GSTM1, GSTTl, XRCC1, XRCC3). With regard to the cytogenetic assessment, the exposed group showed a significantly higher frequency of genetic damage when compared to the control group considering all the cytogenetic parameters evaluated: chromosome/chromatid-type aberrations frequencies and the total of chromosome aberrations in workers (chromosome = 4,2 %, chromatid = 6,9 %, total = 10,9 %) appeared significantly higher ($p < 0.0001$) than in controls (chromosome = 0,8 %, chromatid = 1,8 %, total = CA, 2,7%). Similarly, micronucleus frequencies and DNA primary damage appeared significantly higher ($p < 0.0001$, $p < 0.0002$ respectively) in workers (13,2‰, 0,50 respectively) than in controls (5,2‰ and 0,17 respectively). These results are indicative of a potential genotoxic risk and corroborate the need to maintain safety measures to avoid exposure. This research has been supported by Ministry of Public Health (Progetto di Ricerca Finalizzata, bando 2001: "La valutazione dei rischi nella manipolazione dei chemioterapici antiblastici in ambiente sanitario")

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USE OF CITOMETRY FOR THE ''REPRODUCTIVE MODEL'' ASSESSMENT OF THE SHRIMP PALAEMON SERRATUS (PENNANT, 1777)

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The reproductive biology of the shrimp *Palaemon serratus* (Crustacea, Decapoda), fisheries resources, has been scarcely studied, especially in the marine habitats of Italian waters. For this purpose, in the present study, monthly samples were taken in brackish waters of the Po River Delta in the North Adriatic Sea. The annual maturative cycle of the ovary was assessed both qualitative, on the basis of histological findings, and quantitative terms, by cytometry. The ovaries feature a proliferative centre from which maturing oocytes branch out in a centrifugal direction. The reproductive period falls between March and June. During the non-reproductive period oocyte diameter ranged from 24 to 153 mm, whilst during the reproductive period ranged from 24 to 733 μm. The oviposition pattern may be described as "fractioned," since it is recurrent. In fact, histological and cytometric findings of the Spring catch revealed that the females had both issued eggs that were still attached to the abdomen,

as well as oocytes in all stages of vitellogenesis. The size at first maturity was around 51 mm of length. In conclusion, the histo-cytological approach applied throughout the ovary maturation cycle is a good "reproductive model" assessment and an environmental test because the gonadal maturation is closely dependent on enviromental conditions.

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PLANT CELLS AND FUNGAL TOXINS INTERACTION STUDIED BY FLOW CYTOMETRY: PRELIMINARY DATA ON THE TOXIC EFFECTS OF BEAUVERICIN ON NICOTIANA TABACUM PROTOPLASTS

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Beauvericin, a cyclic hexadepsipeptide, belongs to a group of mycotoxins produced by several species of the genus *Fusarium* that are natural contaminant of cereals. Beauvericin has a broad spectrum of biological activities, in particular it has been reported to possess insecticidal, antibiotic and apoptotic properties. The toxic effects of Beauvericin have been studied by biological assays both in various species of invertebrates (*Artemia salina* larvae and insects) and in mammalian cell lines. Moreover, studies reported that beauvericin was highly toxic to plant protoplasts, but, most of test evaluations are time-consuming, labour-intensive and require considerable ability and training. The phytotoxic effect of beauvericin was evaluated by a biological assay on *Nicotiana tabacum* protoplasts. Protoplasts (at the concentration of 10^5/ml) were exposed to different concentrations of the toxin (10 μM–50 μM and 100 μM) for different times. Flow cytometry analysis was used to evaluate plant protoplasts viability and cell membrane integrity after vital staining with fluorescine diacetate (FDA). After 4 hours of exposure to the toxin, a reduction of 61% of protoplasts viability was observed. Preliminary data on phytotoxicity of beauvericin on plant protoplasts, studied by flow cytometry, will be showed and its usefulness discussed in comparision to traditional methods of toxicity evaluation.

Hematology

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IMMUNOPHENOTYPIC FEATURES OF MESENCHYMAL CELLS (MSCs) CULTURED *UNDER* DIFFERENT CULTURE CONDITIONS

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In this study, different ex-vivo expansion protocols for MSCs were used to test changes in MSC morphology, immunocytochemical pattern expression, immunophenotypic profile by 4-color flow cytometry, growth kinetics, differentiation potential and capability to sustain *in vitro* hemopoiesis. Mesenchymal cells obtained from bone marrow (BM), skin (SF) and from lipoaspirates (LP) were comparatively evaluated. Cultured MSCs were analysed using a gating strategy based on the exclusion of CD457AAD+ (7-aminoactinomycin D) events. Our cytofluorimetric approach allowed the recognition of several mesenchymal cell subsets and was capable of discriminating MSCs from hematopoietic contaminants and dead cells (7-AAD+). Culture conditions could affect the expression of several phenotypic markers (CD90, CD105, CD106, HLA-DR, CD146); in contrast, the reactivity for CD106, CD10, CD34 was found to be dependent on MSC source. Differences in MSC functional capacity (in sustaining LTC-IC assay, and differentiating towards osteogenic and adipogenic lineages) were also observed in relation to the MSC culture conditions as well as to the MSC source. These differences should be considered before MSCs infusion in humans.

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CLONAL EXPANSION CAPACITY AND IMMUNOPHENOTYPIC PROFILE OF CIRCULATING AND BM-DERIVED ENDOTHELIAL PROGENITOR CELLS

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The reactivity of a wide panel of mAbs in cultured PB and BM-derived endothelial cells (ECs) and their progenitors (EPC and CEC) was evaluated by gating on CD45-7AAD-events. The number of PB-ECs (CEC) was very low in patients with hematological disorders, although they displayed an high proliferation rate; BM-ECs (CFU-En) showed a depressed expansion attitude that was found to be strictly dependent on the type of stimulating medium. In some cases, the clonal expansion of CFU-En failed due to excessive apoptosis. Four-colors flow cytometric analysis showed that CFU-En and CEC were mostly negative for CD90, but mostly positive for CD105+/CD31+, CD146+/CD31+, CD29+CD31+; in a small proportion of the cells examined, ECs expressed CD34+/CD31+, CD106+/CD31+, CD166+/CD31+, CD184+/CD31+. CD34+ CEC cells ranged from 40–70% and resulted positive for CD184+, CD105+, CD31+, CD146+, AC133+, while the expression of KDR and CD117 was restricted to less than 30%. The percentage of CD34+ cells was related to culture conditions and timing. The percentage of CD34+ CFU-En resulted lower and ranged from 15–35%. These cells were AC133, and CD117 negative, but positive for the other markers. Interestingly, EC-like cells (ELCs) were frequently detected in PB (especially in

MDS) and BM, they formed EC-like *in vitro* tubular structure but did not show expansion capacity, were positive for CD31+, CD105+, CD45+ CD14+, CD44+, CD68+ thus revealing their monocytic nature. In conclusion, this study proposed a 4-color cytofluorimetric analysis that can identify different EPC subpopulations. Our cytofluorimetric approach resulted successfully for the detection of EC in fresh nonexpanded BM and PB samples.

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ADULT AND CHILDHOOD T-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL)

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T-ALL accounts for 15–20% of new diagnosed cases of ALL. It is characterised by a male predominance, high WBC count, mediastinal and central nervous system involvement and a worse prognosis than other ALL forms. We reviewed our experience about 66 patients with T-ALL (17 children; 49 adults) seen at our Institution between 1985 and 2004. Median age of adult and childhood patients was 22 (range: 16–75) and 9 (range: 4–15) years, respectively. Male/Female ratio was 47/19 (adults 33/16; children 14/3) All cases were classified in three ontogenic stage-related subtypes according to the immunophenotype: Subtype I or early T-ALL (CyCD3+/CD7±/CD1-/CD3): 39 patients (59,1%). Adult/childhood ratio 33/6; median WBC 19x10E9/L (range 1–260); in 14 patient (35,9%) was present a mediastinal mass; CD34 expression was observed in 26/34 cases (76,5%); myeloid antigens (MyAg) (CD13 and/or CD33 and/or CD15 and/or CD65) were coexpressed in 18/35 cases (51,4%). Subtype II or cortical T-ALL (CD7+/CD1+/CD3−): 20 patients (30,3%). Adult/childhood ratio 12/8; median WBC 39x10E9/L (range 7–1000); mediastinal tumor in 13 patients (65%); CD34 was positive in 4/17 cases (23,5%) and MyAg were co-expressed in 1/16 cases (6,2%). Subtype III or mature T-ALL (CD7+/CD1−/CD3+): 7 patients (10,6%). Adult/childhood ratio 4/3; median WBC was 18x10E9/L (range 4–480); mediastinal tumor was seen in 4 patients (57,1%); no case expressed CD34 and MyAg co-expression was only shown in one case (14,3%). On the whole, 51 patients (77,3%) achieved Complete Remission: 35 (71%) and 16 (94%) adult and childhood patients, respectively). Regarding to immunological groups, 27 (69%) early T-ALL, 18 (90%) cortical T-ALL and 6 (85,7%) mature T-ALL patients achieved CR (p 0.035); among these, at present (median follow-up 136 months – range: 5–236), 24 patients are alive in CCR: subtype I: 10 patients (37%); subtype II: 12 patients (66,7%); subtype III: 2 patients (33,3%); p 0.012. Our data confirm the clinical and biological heterogeneity of T-ALL: a lower incidence of lymphomatous features was observed in the less mature subtypes, in which, on the contrary, an higher co-expression of CD34 and MyAg was found. In our experience the immunophenotypic classification was the most relevant prognostic factor in TALL: in adult and in childhood as well, the cortical subtype showed a superior outcome compared to early and mature subtypes.

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EXPRESSION OF ULBPs AND CIRCULATING γδ T LYMPHOCYTES IN LOW GRADE LYMPHOMAS

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It is known that citculating Vδ2 T lymphocytes can kill lymphoma cells, while the Vδ1 subset, mainly resident in the mucosal tissue, is effective against acute myeloid and chronic lymphocytic leukaemia (CLL). Possible targets for γδ T cells in hematological neoplasias are the UL16-binding proteins (ULBPs), non-conventional-MHC molecules related to MIC-A. We analysed 22 patients with low grade lymphomas, 5 mantle (MT), 4 marginal zone (MZ), 13 follicular (FL) (bone marrow and peripheral blood) by flow cytometry and PCR for the expression of ULBPs and for the percentage of circulating Vδ1 or Vδ2 T cells, compared to 4 diffuse large cell lymphomas (DLCL) and 10 high risk (HR) CLL with lymph node involvement. FL lymphomas were $CD5^-$and weakly $CD23^+$ or negative, MT and MZ lymphomas were $CD5^+$ and $CD23^+$ respectively, while DLCL were mostly double negative. B-CLL were $CD5^+CD23^+$ as described. ULBP2 mRNA was transcribed in 8/10 CLL, all MT or MZ and 4/13 FL lymphomas; the protein was expressed at the cell surface in all low grade lymphomas but not in CLL. ULBP3 mRNA was found in MZ and FL lymphomas only, the protein being expressed in all MZ and in 10 out of 13 FL lymphomas, but not in HR CLL. In all low grade lymphomas expressing ULBP2 or ULBP3 or both (18/22) an increase of circulating Vδ1 T lymphocytes, and/or of Vδ2 T cells, was found; conversely in DLCL or in HR CLL, where leukemic cells were ULBPs negative, no γδ T cell increase was found. Thus, a correlation between ULBPs expression and circulating γδ T cells exist in low grade lymphomas. Moreover, FlowCytomix Multiplex analysis of patients' sera for the Th1/Th2 cytokine pattern, revealed increased serum levels of IL4 and/or IL5 in all MT and in 7/13 FL lymphomas, while normal or low levels of IFNγ, TNFα, IL1β, IL6, IL8 and IL10 were found.

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CORRELATION BETWEEN FLOW CYTOMETRIC PATTERNS OF MYELOID MATURATION AND INTERNATIONAL PROGNOSTIC SCORING SYSTEM (IPSS) FOR MYELODYSPLASTIC SYNDROMES (MDS)

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The current international criteria for the MDS diagnostic classification and prognostic scoring (WHO classification and

IPSS) do not take into account the immunophenotyping features. The aim of this work was to evaluate the relationship between an immunophenotypic atypia grading of myeloid maturation patterns and diagnosis & IPSS in MDS. **Methods**: 14 MDS cases were classified according to the WHO and IPSS criteria (see table). The flow cytomeytric myeloid maturation was studied by CD16/CD11b and CD66b/CD11b patterns. The fluorescence intensity patterns were classified into 3 immunophenotypic atypia degrees (IAD): degree 1 = continuous pattern with immunophenotypic maturation asynchronism; degree 2 = continuous pattern with immunophenotypic delayed maturation; degree 3 = discontinuous pattern with maturative blocks. In each of 14 cases the myeloid maturation IAD was compared to the respective diagnosis and IPSS and in 13 cases the IAD was related to the disease progression during a 4 to 13 month follow-up. **Results**: are summarized in the following table (abbreviations are WHO standards)

IAD	progression	IPSS	diagnosis
1	NO	0	RA
1	NO	0	RA
1	AML	0,5	RA
1	NO	0	MDS-U
1	NO	0	MDS-U
1	—	0	MDS-U
1	NO	0	RCMD
3	NO	0	RCMD
1	NO	0	RCMD-RS
1	NO	1	RAEB 1
2	AML	0,5	RAEB 1
1	NO	2	RAEB 2
2	AML	1,5	RAEB 2
3	AML	3	RAEB 2

Conclusions: the myeloid maturation IAD showed a good relationship with diagnosis, IPSS and follow-up outcome. The immunophenotypic delayed or blocked maturation patterns (IAD 2 or 3, respectively) in this study seem unfavourable prognostic factors. A wider study is needed to confirm these preliminary results.

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PROGNOSTIC RELEVANCE OF CD38 EXPRESSION IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

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Since the early report of Damle et al (Blood 1999), several studies confirmed the independent prognostic significance of CD38 expression in B-CLL. However, the threshold of CD38 positivity is not well established (30%, 20% or 7% as cut-off level to better discriminate among prognostic subgroups). More recently, Ghia et al (Blood 2003) showed that the pattern of CD38 expression (homogenously positive, homogenously negative, and bimodal profile), more than the percentage number of neoplastic B-cells co-expressing CD38 antigen, is useful to identify B-CLL patients at risk of disease progression. Fifty-two patients with B-CLL were investigated (30 male and 22 female; mean age 66 yrs; range 37–91 yrs) aiming to evaluate the prognostic impact of CD38 expression in B-CLL patients according to Ghia's suggestions. Twenty-five (48%) patients showed a homogenously negative pattern of CD38 expression, 12 (23%) an homogenously positive one, and 15 (29%) a bimodal profile. No differences were found between the three groups with respect to Rai stages (low, intermediate, high risk) and peripheral blood lymphocytosis. At a mean follow-up of 25 months from diagnosis (range 0–84 months), 29 patients (56%) started therapy (12/25 homogenously negative, 9/12 homogenously positive and 8/15 with bimodal profile). Moreover, a shorter interval time from the diagnosis and the start of therapy was recorded in the bimodal profile group of patients (mean time: 12 months; range: 0–27 months) with respect to homogenously negative (mean time: 33 months; range 0–84 months) and homogenously positive (mean time: 23 months; range 0–67 months). Finally, CD38 homogenously negative patients have a significantly longer survival (median survival 120 months) than homogenously positive (median survival 85 months) and bimodal profile ones (median survival 51 months). The differences among the three curves were statistically significant (p 0.00196; Fig. 1). Significantly different curves (Fig. 2) were also found when patients were stratified according to 30% cut-off of positivity (p 0.0383), while no differences were found using a cut-off of 20% (p 0.1142). In conclusion, in our hands also, the analysis of B-CLL patients according to the expression pattern of CD38, instead of a numerical cut-off, has shown to identify groups of patients with different prognosis.

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DIRECT ANTIGLOBULIN TEST (DAT)-NEGATIVE HEMOLYTIC ANEMIA DURING THE TREATMENT WITH MONOCLONAL ANTIBODY AGAINST CD52 (ALEMTUZUMAB) IN A PATIENT WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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The CD52 antigen is a lymphocyte glycoprotein attached to the cell membrane by a glycosylphosphatidyl-inositol (GPI) anchor. The treatment with the humanized monoclonal antibody Alemtuzumab (Campath-1H), which recognized CD52, resulted in complete depletion of CD52positive cells, including T and B lymphocytes, monocytes and natural killer cells

from the peripheral blood. A 52 year-old man suffering from CLL was referred to us from other Institution to be treated with Campath-1H. He was previously treated with fludarabine and scheduled to receive the monoclonal antibody subcutaneosly three times weekly for 12 weeks at a dose of 10 mg/day as in vivo purging. In May 2003 he started dose escalating Campath-1H (1 mg, 3 mg, 5 mg, 7 mg then 10 mg/day). After six full doses, the patient developed a DAT-negative hemolytic anemia (Hb 8,8 g/dl; LDH and indirect bilirubin serum levels increase). Campath-1H was then stopped and orally prednisone (1 mg/Kg day) was given with hemoglobin recovery and disappearance of laboratory signs of hemolysis. After 3 weeks the patient was re-treated with Campath-1H with the dose escalating schedule previously used. However, after the fourth administration (7 mg), the patient experienced again DAT-negative hemolytic anemia. The drug was discontinued and orally steroids were given with a hemoglobin levels rise and LDH and bilirubin serum levels normalization. After three weeks, to the patient was once again given Campath-1H but after only two doses (3 mg and 5 mg) the drug was definitively stopped because of the reappearance of DAT-negative hemolysis. Finally, the patient returned to his Institution to continue the treatment program. A paroxysmal nocturnal hemoglobinuria (PNH) phenotype in human lymphocytes has been reported as a consequence of anti-CD52 monoclonal antibody therapy. No data are available on the effects on red blood cells so far. Our hypothesis to explain this case is the imbalance of T cells in a patient in which a profound disturbance of immune system is present because of CLL and fludarabine treatment previously given.

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PERSISTENT POLYCLONAL B-CELL LYMPHOCYTOSIS (PPBCL) IN A WOMAN WITH BONE MARROW INVOLVEMENT OF BREAST CANCER

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PPBCL is a well-defined entity characterized by a B-cell lymphocytosis with polyclonal expression of light chain Ig. It is even more frequently observed in smoker women, where lymphocytes are bi-nucleated and the clinical course is favourable. We report on a 53-year-old woman diagnosed and treated (surgery, chemotherapy and tamoxifen) in July 1998 for a ductal carcinoma of the left breast. In April 2003, breast cancer involvement of both cervical nodes and a right axillary node was documented so docetaxel was given and in December 2003, because of a disease progression, capecitabine and exemestan also. In September 2004, thrombocytopenia (50×10^9/l) with spontaneous gingival bleeding, slight anemia (Hb 11.1 g/dl) and lymphocytosis (WBC 13,8 $\times$ 10^9/l; 53% lymphocytes) were observed thus the patient underwent to a re-evaluation of her neoplastic disease. At Hospital admission (November 2004) laboratory was: Hb 8.7 g/dl; MCV 95.6 fL; WBC count 30,1 $\times$ 10^9/l, 60% of which lymphocytes; platelet count $10^6 \times 10^9$/l; LDH 1.747 IU; total/direct bilirubin 1.0/0.7 mg/dl; DAT negative; reticolocyte count 168 $\times$ 109/l; IgG 1,412 mg/dL; IgA 421 mg/dL; IgM 107 mg/dl. Antibodies against HBV, HCV, HIV, TORCH and Epstein-Barr virus were not significant. On peripheral blood film lymphocytes were found as mature appearing. Bone marrow aspiration yelded a hypocellular specimen. Bone marrow biopsy showed diffuse infiltration of epithelioid cells morphologically similar to those present in the primary breast cancer. In the examined biopsy, there were also two small nodular aggregates of mature mononucleated mostly B lymphocytes. The flow cytometric analysis of both peripheral blood and bone marrow samples showed an increase of polyclonal B-lymphocytes, while T and NK-cell number were found normal. Polyclonality of B-cells was also demonstrated by means of the molecular detection of V-D-J sequences. Finally, the cytogenetic analysis showed a normal karyotype. Patient was commenced on Vinorelbine as salvage treatment but died on February 2005 of disease progression. We reported a case of PPBCL with unusual features. The patient was a non-smoker female, polyclonal B-cell lymphocytes were mononucleated, no serum IgM and chromosome aberrations were found, and a striking association with a metastatic bone marrow involvement of breast cancer was observed.

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A CASE OF PEDIATRIC T CELL ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) WITH CO-EXPRESSION OF CD19 IN ASSOCIATION WITH TEL-AML1 REVEALED THE PRESENCE OF ABERRANT TCR GENE REARRANGEMENT

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We report a case of pediatric T cell ALL, where flow cytometry analysis was determinant not only for phenotype of blasts but also revealed anomalous population that further analysis identified carrier of aberrant TCR gene rearrangement. At diagnosis WBC was 330.000/mmc with 90% blasts, bone marrow morphology revealed 90% blasts having two morphological shapes. A three-colour flow cytometric analysis of bone marrow showed blasts population CD45dim, CD7, CD5, CD2, CD3, CD3cy, CD1a, CD4, CD8, CD10, TDT positive, and 20% co-expressing CD19, thus the diagnosis was T-cell ALL according to FAB classification. Evaluation of CD3+, CD4+ and CD8+ suggested populations in a different state of maturation. This unusual pattern induced further studies. To better characterize the T-cell population the evaluation of TCR gene rearrangements were performed by spectratyping assay, a method that analyze TCR b chain repertoire due to CDR3 size eterogeneity. Spectratyping assay revealed 16/24 Vb with unusual TCR gene rearrangements. Moreover

RT-PCR of the most common rearrangements present in pediatric ALL resulted positive only for t(12;21) TEL-AML1, a recurrent rearrangement of B-cell ALL. Further analysis by fluorescence in situ hybridization confirmed the presence of TEL-AML1 rearrangement in 13% of cells. Studies of TEL-AML1 leukemic cells already showed that blast carrying t(12;21) could have aberrant TCR gene rearrangements. Thus we postulate that the population of blasts co-expressing CD19 would carry aberrant TCR gene rearrangement. To test this hypothesis we performed CD19+ positive selection by magnetic beads. Indeed the positive fraction analyzed by spectratyping showed the unusual TCR gene rearrangement previously identified. In this patient the cytometric evaluation of leukemic cells allowed at first the identification of anomalous blast population that required further analysis to be characterized, thus the role of TEL-AML1 as potential inducer of aberrant TCR gene rearrangements in B-cells. This accurate characterization could also be useful in the follow-up and playing a role in Minimal Residual Disease detection for this patient.

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FOUR PEDIATRIC ACUTE BIPHENOTYPIC LEUKEMIA CASES: DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS

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Biphenotypic leukemia represents a clinical and biological entity whose definition does not convey universal agreement. From January 2002 to May 2005 four pediatric acute biphenotypic leukemia cases have been diagnosed at S. Gerardo Children's Hospital in Monza, stated by the score obtained according to EGIL criteria (Bene C et al, Leukemia, 9, 1995). **CASE 1**: B.M., 4 years old. Bone marrow showed 99% of lymphoid blasts, cytochemical myeloperoxidase (cyt-MPO) was positive in 15% of blasts. Immunophenotype showed high expression of B-lymphoid antigens (cyCD79a, CD10, CD19), absence of myeloid markers except for MPO (expressed with cyCD79a on 50% of blasts). Complete remission (CR) was obtained according to LAM BFM'98 protocol. **CASE 2**: B.E., 8 years old. Bone marrow showed 99% of blasts (50% lymphoblasts; 50% myeloblasts). Cyt-MPO was positive on 30% of blasts. Immunophenotype showed coexpression of myeloid antigens (MPO, CD13, CD15, CD33, CD65) and T-lymphoid antigens (cyCD3, CD2, CD7, CD99). CR was obtained with the LAM 2002/01 protocol. **CASE 3**: B.A., 12 years old. Bone marrow showed 80% of blasts, most lymphoid and a smaller part myeloid. Cyt-MPO was positive in 20% of blasts, while immunophenotype showed coexpression of myeloid antigens (MPO, CD15, CD33, CD65) and Tlimphoid antoigens (cyCD3, CD2, CD7). CR was not obtained by BFM LAM '98 protocol, but with Vincristine, Dexametasome and L-Asparaginase therapy and maintained with AIEOP LLA 2000 protocol. **CASE 4**: FV, 5 years old. Bone marrow showed 46% of blasts (60% lymphoid + 40%myeloid). Cyt-MPO was positive in the 10% of blasts, while immunophenotype showed coexpression of myeloid antigens (MPO e CD33) and T-lymphoid markers (cyCD3, sCD3, CD2, CD7, CD99. Remission was obtained with AIEOP LLA 2000 protocol. **CONCLUSIONS**: a) according to FAB criteria, all the 4 cases studied might be classified as Acute Myeloid Leukemia (cyt-MPO > 3%), but only two out of three cases treated with myeloid-oriented induction therapy achieved remission (cases 1, 2 and 3), while one patient treated with LLA induction therapy has responded to this therapy (case 4). b) Two cases with very similar cytochemical and immunophenotypic profile (case 2 and 3) responded to different therapies. c) The current criteria for the definition of biphenotypic leukemia may not be specific for the choice of therapeutic strategies.

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SIMULTANEOUS APPLICATION OF FLOW CYTOMETRY AND RQ-PCR IN THE DETECTION OF MINIMAL RESIDUAL DISEASE IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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The level of minimal residual disease (MRD) in childhood acute lymphoblastic leukemia (ALL), measured during different phases of therapy, represents an independent prognostic indicator with clinical relevance. The most promising methods of detecting MRD are flow cytometric identification of leukemia-associated immunophenotypes and polymerase chain reaction (PCR) amplification of antigen-receptor genes. In order to establish the correlation between the two methods, we simultaneously applied them in 322 BM samples from 161 consecutive patients studied at day 33 and 78 of remission induction therapy of AIEOP-BFM ALL 2000 protocol. Patients were enrolled in two single-institutions (Monza and Padova) involved in an international collaborative BFM-Study. The threshold for qualitative concordance was established at level of 0.01%. Qualitative comparison showed concordant results in 266/322 samples (82.6%, 229/322 contained MRD < 0.01% and 37/322 $\geq$ 0.01%). In 56 cases (17.4%), the two methods yielded discordant results: forty three samples had $\geq$ 0.01% of leukemic cells by PCR, but < 0.01% by flow cytometry (FC), whereas 13 samples had $\geq$ 0.01% of leukemic cells by FC but < 0.01% by PCR. Quantitative correlation analysis (log-levels comparison in 37 positive concordant samples) showed 67% of concordance (r^2 = 0.53). Similar results (overall concordance 79%) were obtained in 1370 BM samples from all AIEOP centers, centralized in Padova and in Monza for FC and PCR MRD study, respectively. Several methodological differences may limit concordance of data obtained by FC and by PCR, including differences in material processing (total nucleated cells versus thawed mononucleated cells after ficolling), denominator of MRD calculation (intact blast cells among total cells in the same sample versus

blast DNA from follow-up sample relative to blast DNA in the diagnostic sample which is considered 100%), and detection limit (1 leukemic cell in 10.000 normal cells versus 1 in 100.000). Both methods have advantages and disadvantages, and the the most appropriated choice should be evaluated within the complex of several factors such as clinical impact of MRD at each time point, available therapeutic options, timing for interventions, technologic and economic resources.

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IDENTIFICATION AND EVALUATION OF MAST CELLS IN A CASE OF MASTOCYTOSIS BY MULTIPARAMETER FLOW CYTOMETRY

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The term "mastocytosis" is used to indicate a group of disorders with abnormal proliferation and accumulation of mast cells in various tissues like bone marrow, lymph nodes, skin, liver and spleen. Flow cytometry can be very useful to study these pathologies, allowing to evaluate the amount of pathologic mast cells and to distinguish them from normal counterpart. For this reason, a case of a patient arrived to hematologist's observation with suspect of mastocytosis is reported. A sample from bone marrow aspiration was collected and a multiparameter flow cytometry study was performed using 3-colours technique and following monoclonal antibodies: CD3, CD4, CD8, CD19, CD16, CD56, CD2, CD33, CD11b, CD15, CD14, CD66b, CD34, CD117, CD11c, CD35, CD59, CD69, CD25, CD45. CD45 was used as an immunological marker to identify main bone marrow populations, while CD117 was recognized as ideal marker for specific identification of mast cells, staining them very highly. However, a counterstaining with CD45 was executed to discriminate mast cells from other CD117+ cells. Multiparameter study displayed that pathologic mast cells showed an aberrant expression of CD2 and CD25, high levels of CD11c, CD35, CD59, CD69 and the absence of CD34. Acquiring 50,000 events for tube, identification and phenotypic characterization of a very little amount (0.1%) of mast cells was performed, showing that flow cytometry represent a very sensitive technique to study mastocytosis.

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STANDARDIZED PHENOTYPIC QUANTITATION OF MYELOID MATURATION STEPS IN NORMAL BONE MARROW USING MONOCYTE AND NK CD16/CD11b EXPRESSION

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Introduction: The diagnostic workup of myelodysplastic syndromes (MDS) includes morphological, cytometrical and cytogenetical analyses. Flow cytometry has been considered as a potential diagnostic tool especially when morphology and cytogenetics gave undetermined or negative findings. The wide quantitative variability of the myeloid cell ontogenical compartments imposes strict rules to establish clearcut boundaries between the various steps, both in normal and MDS subjects. **Materials and Methods:** Twelve bone marrow (BM) aspirates obtained from normal donors candidate to allogeneic BM transplantation were stained with CD45FITC/CD11bPE/CD16PC5 to analyze myeloid maturation steps. The CD11b−/CD16-negative region was established by gating on CD45-neg/SSC_{low} erythroid cells. Monocytes expressed quite stably $CD11b_{high}$ and $CD16_{low}$, whereas NK cells expressed reproducibly $CD11b_{interm}$ and $CD16_{interm}$, and these features have been used to draw standardized boundaries of fluorescence intensity in arbitrary units. Using such internal biological standards, five regions could be established to enumerate percent promyelocytes (CD11b−/CD16−), myelocytes (CD11b+/CD16−), metamyelocytes (CD11b+/$CD16_{interm}$), overall CD16− myeloid cells and mature granulocytes (CD11b+/CD16+) over $CD45_{interm}$/SSC++ myeloid cells. **Results:** Normal subjects displayed the following features: promyelo = 12.7% ± 3.5; myelo = 34% ± 6; metamyelo = 15.8 ± 4.4; granulo = 36.6% ± 8; overall CD16neg = 23.9 ± 11.8. **Discussion:** Normal, healthy subjects in steady state show a moderate variability of myeloid maturational steps, with CVs ranging from 18% to 28%. The highest variability was evident for the overall CD16neg cell compartment (CV = 78%). Such data obtained using a standardized internal biological control of fluorescence intensity in normal subjects should be taken as a starting point to evaluate accurately the abnormal patterns observed in MDS patients.

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PURIFICATION OF BONE MARROW PLASMA CELLS FROM MULTIPLE MYELOMA PATIENTS USING CD138 MICROBEADS AND ''WHOLE BLOOD'' IMMUNOSELECTION

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Purification of bone marrow CD138+ plasma cells in patients with Multiple Mieloma is an essential prerequisite to allow optimal detection of genetic abnormalities (monosomy 13, translocation IgH/FGFR3) through fluorescent in situ hybridization (FISH) analysis of neoplastic cells. To limit cell manipulation and avoid gradient separation steps that may cause the loss of target cells, we investigated a straightforward plasma cell purification strategy consisting of whole blood immunoselection using CD138 microbeads and AutoMACS (Milteny Biotec). Briefly, CD138 microbeads were added to heparinized bone marrow (10 μL/5 × 10^6 nucleated cells). Following incubation (15 min, 4 °C), bone marrow was washed in selection buffer and then double positive selection using AutoMACS was performed. We have analyzed 50 bone marrow samples containing variable percentages of pre-separation CD138+ cells (0.19–29%). Overall, the median recovery of CD138+ cells was 81% (range, 5285%)

and the median purity was 82% (range, 46–99%). A significant enrichment of CD138+ cells (>58%) could also be achieved in patients showing a low bone marrow infiltration (<1%) of CD138+ plasma cells. A successful FISH analysis was performed in all enriched samples. According to our data, whole bloood immunoselection allows a high recovery of CD138+ cells and can be recommended not only for optimizing cytogenetic analysis, but also for molecular studies, genomic or proteomic analysis, or new drugs evaluation.

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SUCCESSFULL ENGRAFTMENT OF AUTOLOGOUS CD34+ STEM CELL TRANSPLANTATION AFTER HIGH DOSE THERAPY AND SINGLE 6 mg ADMINISTRATION OF PEG-FILGRASTIM

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High dose therapy followed by Autologous CD34+ Stem Cell Transplantation (ASCT) is a milestone of treatment program in most of haematological malignancies. Daily subcutaneous injections of G-CSF (filgrastim/lenograstim) at 5 μg/kg/day until ANC > 500/μl are routinely administered from day +1 following ASCT, in order to accelerate hematopoietic recovery and lessen neutropenic complications. Peg-Filgrastim as a single 6 mg fixed dose subcutaneous injection has been shown to have similar efficacy when compared to G-CSF (filgrastim/lenograstim) in the non-transplant setting, but few data are available in the transplant setting. We used Peg-Filgrastim on day +4 following ASCT for 15 patients (M/F 7/8) with myeloma (8 pts) and relapsed or refractory lymphoma (5 NHL, 3 HD). Median age was 56 (range 18–70) and all patients had peripheral CD34+ stem cells harvested after mobilizing chemotherapy (vinorelbine/cytoxan, R-IEV and RGIFOX schedules) and G-CSF (lenograstim 5 μg/kg/day). All patients received standard conditioning high dose therapy regimen (HD-Melphalan, BEAM schedule) and all were evaluated for engraftment (ANC > 500/μl and > 1000/μl, PLT > 20000/μl, > 50000/μl and > 100000/μl) and for neutropenic complications (T > 38°C fever episodes). Median number of days to ANC > 500/μl was 10 (range 915), to ANC > 1000/μl was 10 (range 9–18), to PLT > 20000/μl was 11.5 (range 9–20), to PLT > 50000/μl was 12.5 (range 11–20) and to PLT > 100000/μl was 14 (range 11–36). Median number of fever episodes (T > 38°C) was 0.5 (range 0–2). These data appear similar when compared with those available in literature about routinely use of G-CSF (filgrastim/lenograstim) in the transplant setting (5 mg/kg/day until ANC > 500/μl administered from day +1 following ASCT). In conclusion, single 6 mg fixed dose of Peg-Filgrastim, in our case, was safe and equivalent to G-CSF for accelerating hematopoiesis after Autologous Stem CD34+ Cells Transplantation.

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SEVEN COLOR ANALYSIS OF NORMAL BONE MARROW BY FACSARIA FLOW CYTOMETER: AN EXTENDED AUTOMATED MYELOGRAM

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In spite of the large development of flow cytometry (FCM) techniques, there has been a limited number of studies about polychromatic (>4 colors) detection of normal hematopoietic development. The aim of this study was to identify bone marrow cell populations by FACSAria cell sorter and seven color immunofluorescence. Within each combination, the seven MoAbs were conjugated with FITC, PE, ECD, PE-Cy5, PE-Cy7, APC and APC-Cy7. All combinations included CD34-ECD, CD10PE-Cy5, CD45-PE-Cy7, CD14-APC, CD11b-APC-Cy7. The first two channels were aimed to detect in detail: granuloid maturation (including eosinophils) and NK cells (CD16 and CD13, first tube), granuloid maturation and monocytopoiesis (CD64 and CD13, second tube), erythroid maturation (CD71 and CD105, third tube), plasma cells, myeloid precursors, basophils and granuloid maturation (CD38 and CD33, fourth tube). By using these MoAb combinations we were able to identify the following 20 bone marrow populations: 1. $CD45_{dim}SSC_{low}$ blast cells; 2. CD34+CD38+ blast cells; 3. CD34+CD38− stem cells; 4. promyelocytes; 5. myelocytes; 6. Meta-myelocytes; 7. Neutrophils; 8. Eosinophils; 9. Basophils; 10. immature erythroblasts; 11. intermediate erytroblasts; 12. mature erythroblasts; 13. total mature lymphocytes; 14. NK cells; 15. primitive hematogones; 16. differentiated hematogones; 17. plasma cells; 18. promonocytes; 19. monocytes; 20. macrophages. We analyzed 20 bone marrow aspirates from patients affected by not infiltrating non-Hodgkin lymphoma and solid tumors and compared the results with those obtained by conventional May Grunwald morphology of bone marrow smears. The correlation coefficient was extremely high (R > 0.95) for each comparison. These findings demonstrate that FCM and smear morphology can be used as parallel techniques able to provide consistent results, and that FCM is able to detect higher number of cell subpopulations.

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CHARACTERIZATION OF HUMAN ERYTHROPOIESIS IN VITRO FROM PERIPHERAL CD34+ BLOOD CELLS

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In vitro models of human erythropoiesis are useful to study the mechanisms of erythroid differentiation from BFU-E to mature erythrocytes both in normal and pathological conditions. Most of the commonly available in vitro liquid cultures are from cell lines or limited by the production of few

erythroid cells mixed with myeloid cells. Here we describe a liquid culture method starting from $CD34_+$ enriched cells obtained from peripheral blood by immunomagnetic separation (Miltenyi Biotech, Auburn, CA) that allows the production of pure human erythroid cells. $CD34_+$ enriched cells were cultured for 21 days in the presence of Stem Cell Factor (SCF) and IL3 plus Epo (3U/mL), added at day 0 or 7 with or without Cyclosporin A (Cy: 1 μg/mL). Cells were analyzed at days 0, 7, 14 and 21 of culture. Cell number and viability were determined by Trypan Blue exclusion. Cell morphology was analyzed by May-Grunwald-Giemsa staining; hemoglobin-containing cells were identified by benzidine staining. The cell antigene profile (CD45, CD34 and Glycophorin [GP]), scattercytogram and Propidium Iodide exclusion test (PI) for cell viability were evaluated by flow cytometry. In all the conditions, the highest recovery was obtained at day 14 of culture, while at day 21 half of the cells were dead . The presence of Cy decreased $CD45_+$ cells (2% at day 14 vs 5% w/o Cy). Epo and Cy added at day 0 produced the highest cell expansion (170 fold versus the baseline) and recovery of erythroid cells. Most of the cells in the culture consisted of proerythroblast at day 7 and of mature erythroblast at day 14 (>90%). The results suggest that in 14 days we could reproduce different steps of human normal erythropoiesis from peripheral $CD34_+$ cells, with high recovery of highly purified erythroid cells.

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BONE MARROW MULTICOLOR CHARACTERIZATION OF MYELODYSPLASIA (MDS). A STUDY ON 350 CASES

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In order to define a flow cytometry (FCM) diagnostic protocol for MDS, we studied, in a large series of patients, the following cell markers: 1. Side scatter (SSC); 2. CD16/CD11b pattern; 3. CD16/CD13 pattern; 4. CD64/CD11b pattern; 5. CD14+ percentage; 6. Monocyte immaturity (CD36+CD14- or CD64+CD14−); 7. Monocyte abnormality (CD14+CD56+); 8. CD71+ erythroid cells percentage; 9. CD71 fluorescence intensity; 10. Erythroid immaturity (CD71+CD105+); 11. CD34+ cell percentage; 12. Myeloid blast cell percentage (CD66bCD13+CD33+). We studied 350 patients, affected by MDS (N = 116), myeloproliferative disorders (MPD, N = 48), acute myeloid leukemia (AML) in complete remission (CR) (N = 59), acute promyelocytic leukemia (APL) in CR (N = 23), acute lymphoid leukemia (ALL) in CR (N = 13), megaloblastic anemia (MA, N = 24), hemolytic anemia (HA, N = 10), sideropenic anemia (SA, N = 4), idiopathic trombocytopenic purpura (ITP, N = 30), non-Hodgkin lymphoma (NHL, N = 13), hypoplastic anemia (HyA, N = 10). MDS were classified as refractory anemia (RA, N = 57), RA with an excess of blasts (N = 11), chronic myelomonocytic leukemia (CMML, N = 23), refractory cytopenia with multilineage dysplasia (RCMD, N = 25). Within the MDS group, SSC and CD16/CD13 analysis were the most sensitive indicators of dysgranulopoiesis. Augmented % of monocytes was the most sensitive marker of dysmonopoiesis, while CD14/CD56 coexpression was the most specific one. Increment of immature erythroblasts was the most sensitive indicator of dyserythropoiesis, while percent of CD34+ cells was predictive of acute transformation. Among non-MDS patients, only APL in CR, HA, SA and NHL displayed completely normal results, while AML in CR and MA showed a series of abnormalities. FCM is a powerful tool to identify dysplastic features, but interfering factors (e.g. AML chemotherapy, B12 deficiency, organ failure) have to be excluded to formulate MDS diagnosis.

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CD117 EXPRESSION ON TWO CASES OF BILINEAL ACUTE LEUKEMIA

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We report the finding of 2 cases of bilineal acute leukemia (myeloid and lymphoid as defined by WHO classification) diagnosed at our institution between January 2000 and May 2005. **Case 1**: a 44 year old Caucasian woman presented with anemia, neutropenia and peripheral blastic cells. Her bone marrow specimen showed 90% of pleomorfic blastic cells characterized by small to medium size, round or irregular nuclei, intermediate chromatin, scanty basophilic cytoplasm without granules apart from 2% of blasts with fine granulation. Three percent of blastic cells were positive for Sudan black and the majority was dimly positive for chloroacetate and naphthyl-acetate esterase not inhibited by fluoride. **Immunophenotypic analysis** was positive for cyCD3, CD2, CD7, HLA-DR, CD34, CD117 and TdT on 85% of cells. CD4, CD5, CD8, TCR, CD56, CD10, CD19, CD79a, CD15 were negative. CD33, CD13 and MPO were positive on 10% of cells, not co expressing cyCD3; half of them expressed CD34. RNA was tested by RT-PCR for BCR/ABL and was negative. The patient was treated with a cycle of chemotherapy for myeloid leukaemia without obtaining remission. Bone marrow smear at that time showed atypical blastic cells with very irregular nucleus. Immunophenotype showed 3 pathological populations: T lymphoblast (3%) co expressing CD117, undifferentiated blasts (7%) expressing bright CD34 and CD117 and co expressing CD2 and CD13, myeloblast (15%) expressing dim CD117 and CD34 and co expressing CD2, CD13, CD33, CD14. **Case 2**: a 68 year old Caucasian man presented with anemia, thrombocytopenia and leucocytosis. The marrow specimens showed 90% of monomorfic blastic cells characterized by small to medium size, round nucleus, lacy chromatin with nucleolus, scanty a granulated cytoplasm; 2–3% of blasts were granulated. Four percent of blastic cells were positive for Sudan black. **Immunophenotypic analysis** was positive for CD34, HLA-DR, dim CD13 and CD45 on 85% of cellularity; half expressed CD79a, CD22, CD19 and TdT, half expressed CD117 but no B-lineage markers. T markers, CD56, CD10, and CD20 were negative. MPO was positive on 10% of cells, not

co expressing B markers. RNA was tested by RT-PCR for BCR/ABL and was negative. The patient didn't respond to a cycle of chemotherapy for lymphoblastic leukemia. A new evaluation of bone marrow showed complete infiltration by myeloblast, confirmed by immunophenotype. **Comment**: acute bilineal leukemia is a rare event, in our laboratory we have found only 2 cases out of 318 de novo acute leukemias diagnosed from January 2000 to May 2005. Both cases expressed CD117 (c-kit) which is considered as a marker of immaturity linked to myeloid differentiation. A recent analysis of specificity of c-kit by the EGIL group have found CD117 positive in 100% of undifferentiated leukemias, 67% of myeloid leukemias, 36% of biphenotypic leukemias and 4% of lymphoblastic leukemias. In our laboratory we never detected CD117 on pure lymphoblastic leukemias (0/53 cases). We conclude that CD117-positive lymphoblastic leukemias should be carefully investigated to rule out a biphenotypic or bilineal phenotype.

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ANTI TUMOR NECROSIS FACTOR-α MONOCLONAL ANTIBODY (INFLIXIMAB) THERAPY IN PATIENTS WITH INFLAMMATORY BOWEL DISEASE (IBD): APPLICATIONS AND SIDE EFFECTS

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IBD include two major pathologies, ulcerative colitis (UC) and Crohn's Disease (CD), both characterized by an exaggerated response of the mucosal immune system to stimuli originated from the intestinal flora. Alterations in intestinal permeability and regional and systemic immune responses may play a role in disease outcome. In UC and CD release of Tumor Necrosis Factor (TNF)-α has been invoked as one of the major factor in the chronicity of the inflammation and this sustained production of TNF-α can be supported by plasma endotoxins detectable in a large percentage of IBD patients. In this framework, the role of enteric bacteria in the pathogenesis of IBD is discussed. The availability of Infliximab, a chimeric monoclonal antibody (MoAb) against TNF-α, has represented a therapeutical advance in the treatment of CD patients. In CD patients with moderately active disease, a single infusion of Infliximab led to a significant reduction in their score on a standard CD activity index and one third of the total achieved actual clinical remission. However, the response lasted from a few weeks to six months or more. Same pattern of responsiveness was seen in patients with perianal and cutaneous fistulas. According to some completed trials, serial administration of the drug seems to be more efficacious to maintain the initial response., however this indication has not yet been approved. The mechanisms of action of Infliximab are , then, described and personal data with a cytofluorimetric method for the immune follow-up of CD patients under Infliximab treatment are reported. Finally, among the side effects of Infliximab, cases of active tuberculosis have been described after the initial treatment.

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EVALUATION OF EFFECT OF EUCALYPTUS ESSENTIAL OIL ON MACROPHAGE PHAGOCYTIC ACTIVITY BY CONFOCAL LASER SCANNING MICROSCOPY AND CYTOFLURIMETRIC ANALYSIS

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Many species of the genus *Eucalyptus* from the Myrtaceae family are used in folk medicine for a variety of medical conditions. Monoterpenoid components of the aromatic constituents of the oils are traditionally used as analgesic, antiinflammatory, and antipyretic remedies for the symptoms of cold, flue, and sinus congestion and are commercially available for the treatment of the common cold and other symptoms of respiratory infections. Macrophages constitute one of the primary cellular mechanism of the immune response playing a pivotal role in the detection and elimination of foreign body such as pathogenic microorganisms. According to our knowledge, no data are actually available in literature concerning the influence of essential oil from *Eucalyptus* on cell component of the immune system except for the effect of some cytokine production. In this study we investigated weather essential oil from *Eucalyptus globulus* (EO) is able to affect the phagocytic activity of human monocyte-derived macrophages (MDMs) *in vitro* and of rat peripheral blood monocytes/granulocytes *in vivo*. The evaluation of phagocytic activity was carried out: a) in EO treated and untreated MDMs *in vitro* by confocal microscopy after administration of 1 μm fluorescent beads; b) in rat peripheral blood monocytes/granulocytes, after *in vivo* EO treatement, by cytofluorimetric analysis using the phagotest kit from ORPEGEN Pharma. Our results demonstrate that EO is able to activate MDMs and peripheral blood monocytes/granulocytes both *in vitro* and *in vivo*, stimulating their phagocytic activity. EO

is also able to induce a dramatic recovery of granulocyte phagocytic activity after bone marrow suppression induced by chemotherapeutic agents. Our results warrant further investigations involving components of essential oil extracts from eucalyptus for possible development of a new class of immunoregulatory agents useful in chemotherapy.

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TWO NATURAL MUTANTS OF αIIbβ3 CAUSING CONSTITUTIVE ACTIVATION OF THE RECEPTOR IN GLANZMANN THROMBASTHENIA (GT)

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GT, the congenital deficiency of platelets integrin αIIbβ3, is a very valuable natural model for the study of integrin activation. There is an increasing evidence that a pivotal region for the activation of αIIbβ3 and the subsequent change of shape of the receptor is the one formed by the 4 EGF domains, particularly rich of disulfide bonds. Previously in two patients with GT we found two missense mutations in EGF-2 (C506Y and C508Y), both converting a cysteine to a tyrosine, which effect is the rupture of two highly conserved disulfide bonds. Both mutations were introduced by site directed mutagenesis in a vector containing normal human β3 and coexpressed in human embryonic kidney cells in association with human normal αIIb. By flow cytometry, with antibodies directed against αIIbβ3, we demonstrated that the mutated receptors were expressed significantly less, and so that the mutations identified were able to cause GT. We then assessed the activation state of the mutated receptors by the cytometric analysis of PAC1 binding, an antibody that recognizes αIIbβ3 only in the active form. We were able to demonstrate that PAC1 binding was significantly higher in cells with the mutated receptor than in cells expressing the normal receptor. Almost all the mutated receptors expressed on the membrane of transfected cells were binding PAC1. We could conclude that the breakage of two specific disulfide bonds, C506-C501 and C508-C521, is able to cause the activation of αIIbβ3, so adding evidence to the hypothesis that the central event of the receptor activation is the rearrangements of cysteines bonds.

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PREDICTIVE MARKERS OF CLINICAL EVOLUTION IN KIDNEY TRANSPLANT: A PILOT STUDY

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Vascular, infectious and cancer diseases are serious post-transplant complications in kidney recipients. Oxidative stress in association with lymphocyte activation and immunosuppressive drugs have been evoked in the genesis of the endothelial damage. In order to identify some early prognostic marker, we performed a pilot cross sectional study on 17 recipients (3 of them have neoplasia) of a cadaver donor transplantation. We studied T, B, NK, early and late T-activation markers (CD69+CD3+ and DR+CD3+) and two monocytes markers (CD69+CD14+ and DR+CD14+). Reactive Oxygen Species (ROS) were measured by spectrophotometric method. Statistical analysis was performed by Student *t* test. All recipients showed CD3+DR+ % and ROS higher ($p < 0.001$ and $p < 0.05$, respectively) and B cells lower ($p = 0.01$) than reference values. Recipients affected by neoplasia showed CD3+CD4+ % higher ($p = 0.008$) and NK % lower ($p = 0.007$) than reference values, and CD3+DR+ % higher ($p = 0.017$) than other recipients. The parallelism between oxydative stress and cell activation and the possibility to identify potentially high risk cancer patients, if confirmed by larger studies, could be a step toward the truly tailored immunosuppression.

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DUAL EFFECT OF HUMAN MESENCHYMAL CELLS ON T CELL PROLIFERATION AND SURVIVAL

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Bone marrow mesenchymal cells (MSC) have been shown to play a crucial role in the maintenance of hematopoiesis. They also possess immunosuppressive properties both in vivo and in vitro. We investigated the effects of human mesenchymal cells on T cell activation and functions. PBMC were co-cultured with different MSC suspensions at the ratio of 1:1 and 1:4 in the presence of TCR-mediated (aCD3) stimulus. Cells were cultured for 4 days to assess proliferation of target T cells. MSC strikling decreased T cell proliferation at both ratios and such inhibition was partially restored by co-incubation with rh-IL2 suggesting the induction of T cell anergy. The inhibition of proliferation was not based on the induction of apoptosis as suggested by the low percentage of annexin V positive T cells after co-culture with MSC.To verify the state of T cells upon MSC interaction, we evaluated the expression of Ki67 and we found that MSC decreased Ki67 expression suggesting that most T cells are in the G0 phase. Following IL-2 administration MSC treated T cells started proliferating again supporting their previous state of anergy. Than, we sought to verify whether MSC could support survival and homeostasis of anergic T cells. Indeed, MSC rescue T cells from death when cultured in serum free condition in the absence of any stimulus. MSC also decreased the percentage of annexin V positive T cells upon the induction of activation induced cells death. In such experimental condition, MSC also decreased the frequency of Fas and FasL and granzyme B on stimulated T cells. Altogether, these results suggest that MSC have a dual function on T cells prolif-

eration and homeostasis which appears to be addressed to their survival in a quiescent state.

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HIV GENOME AND LONG TERM NON PROGRESSOR

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Introduction: Five to ten percent of human immunodeficiency virus type 1 infected individuals remain asymptomatic without antiretroviral therapy for more than ten years and present stable levels of CD4+ cells (>500 per mm^3). These individuals are termed long term non progressor. **Case report**: We describe a case of a long term non progressor who has been infected in 1990, who presents stable CD4+ cell counts (~800–1000 cells/mm^3) and viral load (300–500 copies/ml). The aim of our study was to analyse by sequencing the entire proviral HIV1 genome to find out whether gene defects could account for the stable clinical picture of this patient. **Material and methods**: *Extraction of proviral DNA,PCR, Cloning and Sequencing.* DNA was extracted from 3X106 PBMCs isolated from peripheral bloodstream by Ficoll. PCR was performed using specific primers for HIV1 genes. The amplified product was cloned into pGEM-T-Easy vector and sequenced by GenomeLab DTCS_Quick start kit (Beckman Coulter). The obtained sequence was alligned to the HXB2 consensus sequence deposited in Los Alamos HIV Database. **Results**: Clonal analysis revealed gross deletions in *nef* gene, U3 region of LTRs with a conserved TAR sequence, and *env* gene. Sequencing of the remaining genome regions (*gag, pol, vif, vpr, vpu, tat*, and *rev*) showed conserved sequences. **Conclusion**: It is known that *nef* gene may induce down regulation of CD4 and HLA class I and II molecules from the surface of HIV infected cells, which represent an important escape mechanism for the virus to evade an attack mediated by cytotoxic CD8+ T cells and to avoid recognition by CD4+ T cells. The *env* gene delitions fall into the V1 and V2 regions. Whether these defects could affect the protein folding will be assessed by *in vitro* studies. Based on these findings we can hypothesize that the gross defects in *nef* gene along with those found in *env* gene could slower disease progression in our patient.

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REPORT ON CQLINF – EQAS ACTIVITY FROM 1995 TO 2002 (RESULTS OF AN ITALIAN INTER-REGIONAL EXTERNAL QUALITY ASSESSMENT SCHEME ON LYMPHOCYTE IMMUNOPHENOTYPING)

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Results of CQLinf – EQAS from 1995 to 2002 are presented. During this period the number of participating laboratories increased from 29 to 42 (although a more variable % of data returns after each send-out), while the number of specimens (E) per year increased from 6 to 14 (total 70). The laboratory performance criteria was modified (in) according to the criteria developed by the Liguria Region QALI. The inter-lab geometric mean CV% (rCV_{gm}) values and their standard deviations (SD) are summarized in the following table:

1995–2002

	TCD3		TCD4		TCD8		B		NK		WBC	Ly	
n̂ E = 70	%	#	%	#	%	#	%	#	%	#	#	%	#
rCV_{gm}	3.42	8.25	5.65	9.70	7.51	11.42	19.11	22.51	16.74	19.91	5.77	7.08	7.69
SD	1.19	1.94	2.20	2.49	3.45	3.32	9.36	8.42	8.59	8.80	2.23	2.47	2.45

The CQLinf – EQAS exercises were performed by fresh peripheral blood samples analysed within 48 h. A preliminary study demonstrated the stability of samples over 5 days. Initially the participant laboratories performed the results by double platform (dp), from 1999 an increasing number of laboratories perfomed results by single platform (sp). This occurrence allowed an inter-laboratory variability comparison between single and double platform in 40 exercises performed on fresh peripheral blood. The CV_{gm} values and their SD are summarized in the following table:

CD3	TCD4	TCD8	CD19	CD16
sp dp	sp dp	sp dp	sp dp	sp dp
rCV_{gm} 7,40 7,83	7,45 9,20	8,36 11,04	15,51 22,53	18,29 19,21
SD rCV_{gm} 2,43 1,86	3,41 2,59	5,56 4,54	10,16 8,74	17,39 9,02

These results demonstrate a good agree between single and double platform. Other statistic methods were applied to compare single and double platform, the Bland & Altman difference analysis and regression line and finally the TCD4 results regression line was drawn into the acceptability boundaries by A. Kunkl. The results will be analytically presented. **Conclusions**: overall statistic results confirm a good agree between single and double platform using fresh peripheral blood samples. Moreover the comparison between rCV_{gm} from overall 70 and the last 40 samples by both single and double platform suggests that implementation of CQLinf EQAS had an educational role.

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PERFORMANCE OF ABSOLUTE TCD4+ CELL COUNTING BY A LEUCOGATING DUAL PLATFORM METHOD IN AN EXTERNAL QUALITY ASSESSMENT SCHEME (EQAS) ON LYMPHOCYTE IMMUNOPHENOTYPING

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Previous works demonstrated that absolute TCD4+ cell counting by a leucogating dual platform technique, displayed a good performance as compared to the standard method based on lymphocyte gating flow cytometric

analysis. The aim of this work was to evaluate the performance of absolute TCD4+ counting by a leucogating dual platform method in CQLinf-EQAS on lymphocyte immunophenotyping. **Method**: the results of TCD4+ cell counting by leucogating dual platform retrospectively obtained from 30 consecutive CQLinf exercises (E) were compared with the respective counting obtained by the standard lymphocyte gating analysis method. The performance of both methods was evaluated and compared by the CQLinf-EQAS scoring system (S) (0 point = inadequate, 1 point = adequate, 2 points = good) and by acceptability boundaries of residual limits analysis described by A. Kunkl. **Results**: are summarized in the following table:

	n° E with S=2	n° E with S=1	n° E with S=0
	(% on total E)	(% on total E)	(% on total E)
(WBC-gate)/mL	22 (73,33%)	6 (20,0%)	2 (6,67%)
TCD4 (Ly-gate)/mL	22 (73,33%)	3 (10,0%)	5 (16,67%)

The regression lines of residual values of both methods were within the boundaries of acceptability. **Conclusions**: absolute TCD4+ cell counts by a leucogating dual platform method retrospectively extracted from CQLinf-EQAS showed an almost identical score as compared to standard analyses. A simplified TCD4+ cell counting approach can be therefore validated for diagnostic use thanks to the CQLinf EQAS scoring system. A further strengthening of our approach may derive from a similar multicenter retrospective analysis among CQLinf-EQAS users.

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MODIFIED EXPRESSION OF BCL-2 AND SOD1 PROTEINS IN LYMPHOCYTES FROM SPORADIC ALS PATIENTS

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Amyotrophic lateral sclerosis (ALS) is a progressive disorder characterized by motor neuron degeneration in the brain and in the spinal cord. The majority of patients are affected by the sporadic form (SALS) of the disease, whose aetiology remains unknown. Markers of oxidative stress have been found in spinal cord, cortex, cerebrospinal fluid, and plasma of SALS patients. Previous data obtained in our laboratory showed the presence of mitochondrial and calcium metabolism dysfunctions in lymphocytes from SALS patients. In this study, we demonstrated that lymphocytes from SALS patients are more damaged than lymphocytes from age- and sex-matched controls, both in basal conditions and following oxidative stress induced by H_2O_2 treatment. We also investigated the role of the Bcl-2, SOD1 and catalase proteins (all involved in the oxidative stress pathway) by comparing the expression of these proteins in lymphocytes from SALS patients and controls in basal conditions and after oxidative stress. In basal conditions, the expression of Bcl-2, SOD1 and catalase was found to be significantly lower in the lymphocytes from the SALS patients. Oxidative stress produced different expressions of the antioxidant proteins in the control and SALS lymphocytes. Exposure to H2O2 induced a time-dependent decrease of Bcl-2 and SOD1 in the control lymphocytes. Conversely, the levels of these proteins remained unchanged in SALS lymphocytes even after 18 h stress. Catalase expression was not significantly modified by oxidative stress. Thus, the oxidative pathway is deregulated in lymphocytes from ALS patients, indicating that traits of the neurodegenerative process are also present in peripheral blood cells.

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ESTABLISHMENT OF NOVEL HUMAN NEURAL STEM CELL LINES

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A renewable source of human brain cells bearing all the features of neural precursors would be of immense value for both basic and preclinical studies. By v-myc, we established immortal cell lines from various regions of fetal human brain, which have the capacity to generate unlimited amounts of mature neurons, astoglia and, differently from previous studies, significant numbers of oligodendroglial cells. The average doubling time of these cell lines was less than 36 hours, with well over 25% of the cells found in S-phase (BrdU pulse of 23 minutes). Accordingly, immortalized lines displayed a sensitivity to camptotecin that was an order of magnitude higher than normal human precursor cells. Neither kariotype mutation nor loss of growth factor-dependence were ever observed over extensive passaging (over passage 100th). The neurons generated from these cells expressed al appropriate antigenic markers and were able to form active synapses, as shown by anti-p38 immuno reactivity. The most representative, detectable neuronal neurotransmitter phenotypes were the GABAergic, cholinergic and glutamatergic ones and glutamate receptors were also observed. Electrophysiological analysis and transplantation experiments are underway to determine the ability of these neurons to elicit action potentials and to integrate as bona fide neurons in vivo. Characterization of v-myc clonogenic cells showed that they express CD9, CD29, CD44, CD49e, CD49f, NGFR(dim), CD81, CD90 (dim) and PDGFRa but not CD11a, CD18, HLADR, CD51/61, CD34, CD49a, CD11b, CD49c, CD49d, CD117 and PDGFRb. Thus, we describe new, continuous cell lines of human multipotent precursors that are safely expanded in culture, with no sign of transformation and retaining the features of normal neural cells, providing a novel model for the development

of high throughput assay for both drug discovery and basic studies on neural cell development.

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CIRCULATING ENDOTHELIAL CELLS ARE INCREASED IN PATIENTS WITH CLASSICAL KAPOSI'S SARCOMA

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Kaposi's sarcoma (KS) is multifocal highly vascularized neoplasm characterized by the presence of spindle-shaped tumor cells, angiogenesis, and inflammatory infiltrates. Four clinical variants of KS have been identified sharing common histologic features and strong association with human herpesvirus-8 (HHV8) infection, but are differentiated on the basis of epidemiological, clinical and prognostic criteria. Among these variants, classical KS (cKS), that is more common among elderly men from Mediterranean countries, represents the best model to study HHV8-associated cancer without the interference of other virological or iatrogenic factors. The pathogenesis of KS is complex: in addition to the involvement of HHV8, immune dysregulation and increased expression of inflammatory cytokines have been suggested to play key roles. Early theories of tumor angiogenesis suggested that preexisting vessels surrounding the tumor were the principal source of the tumor vasculature, but recent evidence suggests that circulating endothelial cells migrating from the bone marrow may play an important role in developing tumor vessels. Circulating endothelial cells comprise at least two distinct populations: circulating endothelial progenitors (CEPs), derived from the bone marrow; and mature circulating endothelial cells (CECs), that may derive both from maturation of CEPs and from existing vasculature. Increased numbers of CEPs and CECs have been described in a number of human cancers. In the present study we analyzed the number of CEPs, identified as CD34+/VEGFR2+/AC133+ cells, and CECs, identified as CD34+/VEGFR2+/AC133- cells, in patients with cKS. Our results indicate that CEPs and CECs are increased in cKS patients, suggesting that dysregulation of endothelial cell proliferation and differentiation may represent a further key factor in KS pathogenesis. The state of activation, HHV8-infection and functional properties of CEPs and CECs were also evaluated.

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DIFFERENTIAL EXPRESSION OF KIR AND CLIR BY Vδ1 AND Vδ2 T LYMPHOCYTES IN HEALTHY DONORS AND HIV-1 INFECTED PATIENTS

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Killer Ig-like receptors (KIR) and C-type lectin inhibitory receptors (CLIR) have been described in natural killer cells, were they can exhibit inhibiting or activating functions, depending on their cytoplasmic tail. KIR and CLIR ligands are represented by HLA-I and its alleles. Recently, it has been shown that also $CD8_+$ γδ T cells, can express either CLIR or KIR. In this study, KIR distribution in Vδ1 and Vδ2 T cell subsets and intracellular IFNγ or IL4 have been analyzed by three color flow cytometry in healthy donors and HIV-1 infected patients. Thirty HIV-1infected patients, in the stage A of the disease, have been studied. We found in 20 out of 30 patients an increase in the number of circulating Vδ1 T cells (3–9%) of T lymphocytes (healthy donors: 1–3%). Three of these patients also displayed an increased number of peripheral Vδ2 T cells (4–6% vs 2–3% in healthy donors). We found that 50% of Vδ1 T cells co-express KIR in healthy donors, while Vδ2 T lymphocytes are mainly $CLIR_+$. In HIV-1 patients, Vδ1 T cells were mostly KIR_+. Interestingly, only Vδ2 $CLIR_+$ T lymphocytes can produce IFNγ in response to PWM and ionomycin in healthy donors, while in HIV-1 patients also Vδ1 KIR_+ T cells were able to produce this cytokine. No production of IL4 was observed by either Vδ1 or Vδ2 T cells, in healthy donors and in HIV-patients. We propose that the increase in Vδ1 KIR_+ IFNγ -producing T cells observed in in HIV-patients are due to the viral infection.

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STUDY OF HUMAN CD4+CD25+ T REGULATORY LYMPHOCYTE FUNCTION IN VENOM IMENOPTERA ATOPIC PATIENTS BEFORE AND DURING ALLERGEN IMMUNOTHERAPY

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The patophysiology of asthma and allergic diseases was dominated by the Th1/Th2 paradigm. Since the early 1970s different subtypes of regulatory and suppressor cells, that may play a role in peripheral tolerance, have been demonstrated, and their biology has been the sub ject of intensive investigation. Whether Tregs normally prevent atopic sensitization and how this regulatory pro cess becomes defective or bypassed in those individuals who developed allergic disease are areas of much cur rent research. Additionally the allergen immunotherapy modifies Th2 response to the allergen, either by Th1immune deviation either induction of IL-10-producing Tregs. Aims of this study were: a) whether CD4+CD25+ T regulatory lymphocyte (Tregs) function normally in atopic patients; b) the follow-up of patients subject to immuno therapy (SIT) with venom imenoptera. The preliminary results don't support a general deficiency in functional CD25+Tregs in allergic patients compared with healthy controls. However during the challenge with allergen the CD25+Tregs are deficient in suppressing Th2 cytokine production. This might be explained by the fact that increased levels of the Th2 growth factor IL-4 inhibit the suppression by CD25+Tregs of Th2 clones. Understan ding of regulatory mechanism in the development of allergic sensitization and their manipulation by

immunothe rapy holds the promise of vaccination strategies to pre vent allergic diseases.

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EVALUATION OF PLATELET GPIIb/IIIa RECEPTOR BOCKADE BY ANTAGONIST

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Inhibition of soluble fibrinogen binding to activated platelet is the target of tirofiban and abciximab, GPIIb/IIIa complex antagonists: drugs used to prevent thrombotic complications of percutaneous coronary interventions. In this study we used a whole blood flow cytometric assay (MoAb CD41 and PAC-1) to evaluate the effect of tirofiban or abciximab therapy in patients with coronary artery disease undergoing to Percutaneous Transluminal Coronary Angioplasty. The patients where treated with aspirin, heparin and gp IIb/IIIa antagonists according to the following scheme: Tirofiban:

- bolus of 25 micro g/Kg
- continuous infusion with 0.15 micro g/Kg/minute. We studied 20 patients before treatment, 15 minutes and 3 hours after bolus infusion Abciximab
- bolus of 0.25 mg/Kg
- continuous infusion with 0.125 micro g/Kg/minute We studied 7 patients during and/or after treatment We have also evaluated platelets function measuring Pselectin (CD62 P) exposition after in vitro stimulation with agonist. As controls we studied 20 healthy volunteers (basal conditions). The percentage of receptors blocked by tirofiban had range of 50–94% at 15 minutes and range of 70–97% at 3 hours after treatment. Two patients had 83–94% of effect at 15 minute and <70% at 3 hours after bolus infusion. The percentage of receptors blocked by abciximab had range of 60–95% during treatment, 24 hour after treatment all receptors were free. All the patients platelets showed normal exposure of Pselectin in basal conditions ad during tirofiban or abciximab treatment. These preliminary data confirm the interindividual variation in response to GPIIb/IIIa antagonist therapy and indicate that the described flow cytomety technique could be a suitable method for assessing the effects of GPIIb/IIIa antagonist and for monitoring these therapy.

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ABSOLUTE COUNT OF PERIPHERAL NK CELLS IN IVF TREATMENT

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Background: Previous studies have suggested that enhanced peripheral NK cell activity or elevated NK cell numbers may be associated with recurrent miscarriage. Recently some authors (1,2) examined the phenotype of peripheral blood NK cells in women with a history of RPL or infertility of uncertain etiology. Expression of CD69, an early activation marker, was significantly higher on NK cells from women with RPL compared with controls. In this study, we investigated prospectively the expression of activating receptor CD69 in peripheral blood NK cells, and expression of $CD4+CD25_{britght}$ of selected women who underwent IVF treatment. Blood samples were obtained prior the procedure, at the time of embryo transfer and after two weeks if pregnancy is established. **Methods:** 100 μl of heparinized blood samples were stained with appropriate mixture of CD45 APC, CD3PerCp, CD4PE, CD8FITC, CD25PE, CD56PerCp, CD16FITC CD69APC, prior analysis 100 ml of fluorescent microspheres were added to each tube. The absolute counts of $CD56_{dim}CD16+CD69+$, $CD56_{bright}CD16-$ NK cells and $CD4+CD25_{bright}$cells were analyzed by multiparametric flow cytometric analysis. Dual laser FACSCalibur (BD) and CELLQuest software with a gate strategy were used to identify NK subsets. **Results:** Data were recorded and their relation to IVF treatment outcome and miscarriage rate was analysed. Preliminary results have shown an increase in the absolute count of activated NK cells ($CD56_{dim}CD16+CD69+$) in the peripheral blood that is associated with a reduced rate of embryo implantation in IVF treatment, in agreement with other authors.

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CONTRIBUTION OF CD8+ CELLS TO DECREASED LEVELS OF LYMPHOCYTE APOPTOSIS IN RESPONSE TO HIGHLY ACTIVE ANTIRETROVIRAL THERAPY IN HIV PATIENTS

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We and other authors have previously demonstrated a prompt inhibition of lymphocyte apoptosis in patients infected with human immunodeficiency virus 1 (HIV-1) undergoing highly active antiretroviral therapy (HAART). However, the contribution of the CD4+ and CD8+ subsets in the overall decrease in lymphocyte apoptosis, has not been fully elucidated. To address this point, HIV-1-infected patients undergoing HAART were enrolled in a long-term, open longitudinal study. Sustained levels of apoptosis inhibition in peripheral blood mononuclear cells were measured, in the long-term, in 16 of the 17 patients with successful response to therapy. Interestingly, in the same subgroup, levels of total cell apoptosis are correlated with levels of CD8+ apoptotic cells

more significantly than with levels of CD4+ apoptotic cells. In addition, in the same subgroup, CD4+ cell counts were correlated inversely with levels of CD8+ apoptotic cells in a highly significant fashion, but not with levels of CD4+ apoptotic cells. Our data suggest that the increase of CD4+ lymphocytes in HIV patients, as a consequence of successful response to HAART, is poorly or not dependent on the inhibition of CD4+ T cell apoptosis, but rather may be related to changes occurring in the CD8+ T cell compartment.

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IMMUNOPHENOTYPE CHARACTERIZATION AND CELL CYCLE ANALYSIS OF MURINE NEURAL STEM CELLS

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The subventricular zone (SVZ) of the lateral ventricles is one of the adult brain regions with the highest neurogenic activity. Under appropriate conditions (like the presence of specific growth factors) it's possible to propagate NSCs from SVZ as neurospheres. We characterized murine NSCs isolated from SVZ of three different mouse strains (BALB/6, C57BL/6, CD-1) for the expression of a complex series of proteins:

- markers of stem cells originary from other tissues than neural (Sca-1, CD90, CD117, CD9 and CD105)
- adhesion molecules involved in cell-matrix and cell-cell interactions (CD29, CD49d, CD49e,CD61, VLA4, CD44 and CD54, E-cadherin, N-cadherin, cadherin 5, M-cadherin, P-cadherin, R-cadherin) and main catenins that interact with cadherins (p120 and betacatenin)
- selectins (CD62L and CD62P) that play a fundamental role in the ematopoietic stem cells adhesion/migration process.

NSCs were all positive for CD9, CD29, CD49e and CD44, E-cadherin and N-cadherin, p120 and beta-catenin and negative for CD117, Sca-1, CD105, VLA-4 , CD54, CD62L and CD62P. Other molecules did show a specific pattern of expression dependently on the strain of origin of the cells (like CD90, CD49d, CD61). In addition we analyzed cell cycle of these NSCs by pulse labeling with BrdU and apoptosis sensitivity to different treatments (camptothecin and Taxol) by TUNEL and caspase3 active staining. The three strains did show different duplication times (TD) and a specific distribution of cell cycle phases. Analogously, they resulted differently sensitive to the camptothecin treatment (confirmed by TUNEL technique). From these data we can conclude that it's essential to study NSCs from different mouse strains to identify a NSC marker independently from the genetic background. In the next future, side population staining in parallel with immunophenotyping staining could be very useful for a complete characterization: successful results will allow to sort "real stem cells" by flow cytometer/cell sorter.

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IDENTIFICATION OF A CONFORMATIONAL MUTANT ISOFORM OF p53 IN BLOOD PERIPHERAL CELLS FROM ALZHEIMER'S DISEASE PATIENTS

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by irreversible cognitive and physical deterioration. AD is morphologically characterized by abundant accumulation of b-amyloid in the form of senile plaques and abnormally phosphorylated tau protein in the form of neurofibrillary tangles. Nowadays, the identification of biological markers of AD, in addition to increase our understanding of the pathogenesis of the disease, can be extremely useful to improve diagnostic accuracy and/or to monitor the efficacy of putative therapies. In this regard, peripheral cells may be of great importance, because of their easy accessibility. Rationale: Among extra-neuronal tissue, cultured skin fibroblasts derived from AD patients have gained particular attention since they show a number of abnormalities in metabolic and biochemical processes, with some of them mirroring events that occur in the AD brain [1]. Recent evidence demonstrated an impairment of p53 signalling pathway following an oxidative injury that resulted in a lower vulnerability of AD fibroblasts [2]: fibroblasts from sporadic AD patients specifically express an anomalous and detectable conformational state of p53 (mutant p53) that makes these cells distinct from fibroblasts of age-matched non-AD subjects.

On the basis of these evidence, in this work (based on the use of flow cytometry integrated by fluorescence microscopy) preliminary data are presented about the different expression of mutant p53 between AD and non-AD subjects, using, as cellular model, blood peripheral cells. These preliminary results, independently from their relevance in understanding the etiopathogenesis of AD, may be a useful tool and a putative diagnostic marker to discriminate AD from non-AD patients.

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IN VITRO PROLIFERATION OF NAÏVE B CELLS AND MEMORY B CELLS: DIFFERENT PROFILES FROM HEALTHY AND HCV-INFECTED INDIVIDUALS

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There is a growing interest in the tendency of B cells to change their functional program in response to overwhelm-

ing antigen loading, perhaps by regulating specific parameters, such as efficiency of activation, proliferation rate, differentiation to antibody secreting cells, and rate of cell death in culture. We show that chronic hepatitis C patients carry low levels of circulating memory B cells. This finding can be explained by the unconventional activation kinetics and functional responsiveness of the memory B cell subset in vitro. Following CD40-mediated stimulation in the absence of B cell receptor (BCR) engagement, memory B cells do not expand, but rapidly differentiate to secrete immunoglobulins and then die. We propose that their enhanced sensitivity to BCR-independent bystander T cell help serves as a backup mechanism of feedback inhibition to prevent exaggerated B cell responses that could be the cause of significant immunopathology.

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STUDY OF THE RELATIONSHIP BETWEEN HIV REPLICATION AND CELL CYCLE IN HIV INFECTED PBMC BY HIGH RESOLUTION FLOW CYTOMETRY

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Multiparametric flow cytometry was used to evaluate the correlation between HIV replication and cell proliferation or activation in HIV infected PBMC and to discriminate the timing of HIV expression during the cell cycle. PBMC were stimulated and infected with HIV-1-NL4.3 or HIV-1NL-r-HSAS (derived from HIV-1-NL4.3 by cloning the vpr region with the murine CD24 – sequence, wich is then expressed on the surface of actively replicating HIV cells). HIV-1-NL-r-HSAS replication was measured as the percentage of mCD24 positive cells. Intracellular staining with anti-p24 – PE was used to determine HIV-1NL4.3 infected cells. Cell proliferation and activation were studied by CFSE staining and by CD69 expression. Cells were analyzed using an EPICS XL flow cytometer (Coulter Electronics); acquisition was performed with at least 50,000 total events. Multicolor flow cytometric analysis allowed us to discriminate cells that had undergone at least one round of DNA synthesis (s-phase) during 24 h BrdU labeling in terms of BrdU incorporation and their cell cycle distribution (G0/G1, S, G2/M phases) defined by 7-AAD staining intensities. (BD PHarmingen). To further correlate HIV-replication with cell proliferation we employed two cytostatic drugs: hydroxyurea (HU) and mycophenolic acid (MPA), which inhibit cell proliferation but not activation by specifically blocking cell cycle progression between the G1 and S phases. S-phase percentage varied from 25.7% $\pm$ 16.0 in untreated samples, to 3.3% $\pm$ 3.3 in HU treated cells or to 13% $\pm$ 2.0 in MPA treated cells.This method of study let us to address the unanswered question of whether cell activation is sufficient or cell proliferation is required to sustain HIV infection and to identify the cell cycle status required for HIV replication.

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SINGLE PLATFORM ABSOLUTE ENDOTHELIAL CELL COUNTING IN PERIPHERAL BLOOD

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Circulating endothelial cells (CEC) have been described in different conditions with vascular injury. We developed a 4-color method and a single platform enumeration protocol to assess CEC and their progenitors (EPC), as markers of endothelial dysfunction. Blood is bulk lysed in ammonium chloride and 100 µl leukocytes (2×10^6) are incubated in TruCOUNT tubes with: 1) IgG1/IgG1/CD45/IgG1, 2) CD146/CD133/CD45/CD34, 3) CD45/CD146/7-amino-actinomycinD (7-AAD). An acquisition threshold is established on FL1 in a FL1 versus SSC plot. Acquisition is stopped when 25.000 TruCOUNT beads, corresponding to half of the original sample, are acquired. Total CEC (CD146+CD34+CD45-) and EPC (CD146+CD133+ CD34+CD45-) events are determined following a series of logical gates in CD45/SSC, CD146/CD45, FSC/SSC, CD146/CD34, CD146/CD133 dot plots and referred to total number of beads to calculate absolute counts. Assay is characterized by these parameters: 1) bead concentration roughly corresponds to 500/µl original sample, 2) sample concentration is 20×10^6/ml, 3) total cell/bead event ratio ranges 2–6, 4) the presence of a minimum of 15 events displaying similar phenotypic and physical features is required to detect a CEC population, 5) the lower limit of sensitivity is 300 CEC/ml leukocytes and 100 CEC/ml peripheral blood. These criteria allowed CEC and CEP identification in 11/15 and 7/15 normal donors respectively with a 40% inter-assay difference.

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FOLLOW UP OF PARIETARIA POLLEN SPECIFIC IMMUNOTHERAPY BY IMMUNE SERUM MEDIATED INHIBITION OF A FLOW CYTOMETRIC BASOPHIL ACTIVATION TEST (FAST)

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We investigated the effects of Parietaria pollen specific immunotherapy by a FAST assay based on CD63 expression induced by crude Parietaria Judaica (PJ) extract or by the recombinant Par j2 protein (rPj2). A cycle of immunotherapy consisted of four pre-seasonal injections of mono-phosphoryl lipid A-adjuvanted tyrosine absorbed allergoid vaccine. Eleven patients were tested before or after the first and the second cycle of immunotherapy. Serum samples were collected before immunotherapy (PRE), three weeks (POST 3w) or 1 year (POST 1y) after last injection of the first cycle and three weeks (POST2 3w) after last injection of the second cycle of therapy. FAST consisted in sti-

mulating 3-week POST whole blood with PJ or rPj2 (100 and 10 ng/ml) pre-incubated with PRE or POST sera and measuring %CD63+ CD123+ HLADR- basophils. Inhibition of activation, due to preincubation with sera, was calculated by taking as 100% the activation induced by allergens alone. Patients were evaluated clinically for a change in allergic symptoms by an overall assessment score (OAS). A significant inhibition of activation was shown when allergens were pre-incubated with serum POST 1y (p 0.032) or with serum POST2 3w (p 0.0576), while inhibition by POST 3w was not significant. A significant correlation was observed between POST 1y and OAS (p 0.03) suggesting FAST can objectively describe the beneficial effects of immunotherapy otherwise defined by more subjective criteria.

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A STUDY OF REGULATORY POPULATIONS IN HUMAN MELANOMA

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The persistent and progressive growh of tumors seems to indicate that the immune system usually is not competent to control the malignancies. On the other hand, the identification of tumor reactive cytotoxic lymphocytes in the peripheral blood of cancer patients suggests the occurrence of an anti-tumor response. In this context, the presence of low immunogenic tumor antigens, a failure in the co-stimulation, as well as the down-modulation of surface MHC molecules, have been referred as relevant for tumor escape from immune rejection. Recent reports consistently indicate the involvement of cell dependent mechanisms in the regulation of the T cell activation/tolerance balance. In this contest, a role for CD4+ CD25+, as well as for natural T (NKT) lymphocyte populations, has been suggested. Our study is addressing the analysis of such populations in the peripheral blood, as well as in metastatic nodes obtained from melanoma patients at different disease stages. Our preliminary data, obtained by comparative analysis of 31 melanoma patients with a group of 10 age/sex matched healthy controls, show a significant increase in the frequency of the NKT in 7 melanoma patients (22.5%), while no significant variation in the CD4+CD25+ subset has been observed. All the patients with an increased NKT population show a metastatic disease. In addition, a correlation between interferon gamma production in the node lymphocytes and the consistency of the metastatic invasion has been observed.

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MODE OF ACTION OF KLH IN DENDRITIC CELL ACTIVATION

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Keyhole limpet hemocyanin (KLH) is a high molecular mass hemocyanin derived from the marine mollusc *Megathura crenulata* native of the Pacific coast of California and Mexico. Although KLH biochemical structure has not been completely clarified, it is widely accepted that its sugar components may play a key role in conditioning the antigenic properties of the intact molecule. The carbohydrate content of KLH has been calculated to represent about 4% of the molecular mass. *In vitro*, KLH is commonly used to evaluate primary Agspecific T cell responses. In fact, its large size makes it very immunogenic. Clinically, KLH is used as a carrier for vaccines and antigens. The use of KLH as a hapten carrier for small molecules, such as many chemicals, drugs, hormones and peptides, has extended the applications of this protein within the broad field of immunobiology and immunochemistry. KLH also acts as a nonspecific immune stimulant when used as a conjugate vaccine. However, the mechanisms by which KLH exerts its immunostimulant activity are still largely unknown. In particular, although dendritic cells (DCs) play a central role in initiation and activation of immune responses, the effects of KLH on these cells have been poorly investigated. The aim of the present study was to investigate the effects of KLH on DCs. To this purpose, monocyte-derived DCs (mo-DCs), obtained by culture of monocytes in the presence of GM-CSF and IL-4, were treated with different doses of KLH. Our results indicate that KLH is able to promote DC activation and maturation, as assessed by upregulation of surface expression of the costimulatory molecules CD80, CD86, and CD40, and of the maturation marker CD83. Moreover, KLH promote the production of the stimulatory cytokine IL-12 by mo-DCs. All these effects are dose-dependent. Because the uptake of antigens through mannose receptor (MR), a molecule expressed on DC surface that recognises carbohydratic residues, represents an important way of DC activation, we further investigated whether the effects of KLH are at least in part mediated by MR. To this purpose, inhibition experiments were performed by use of mannan, which is a natural ligand of MR.

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ALLELISM OF MEDULLARY CYSTIC DISEASE, FAMILIAL JUVENILE HYPERURICEMIC NEPHROPATHY AND GLOMERULOCYSTIC KIDNEY DISEASE CAUSED BY DELAYED UROMODULIN TRAFFICKING TO PLASMA MEMBRANE AND ENDOPLASMIC RETICULUM RETENTION

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The disease complex medullary cystic disease/familial hypeuricemic nephropathy (MCKD/FJHN) is characterized by alteration of urinary concentrating ability, frequent hyperuricemia, tubulo-interstitial fibrosis, cysts at the cortico-medullary

junction and renal failure. MCKD/FJHN is caused by mutation of the gene encodings Uromodulin (also referred to as Tamm-Horsfall protein) and expressed by epithelial cells of the thick ascending limb (TAL) of the loop of Henle and by distal convoluted tubules (DCT). Uromodulin is the most abundant protein in urine and it has been hypothesized to be responsible for water impermeability of the TAL due to its ability to form a reversible gel-like structure and to modulate the urothelial defense against infection. In this study we analyze new missense study in three families with MCKD/FJHN and demonstrate allelism with a glomerulocystic kidney disease (GCKD) variant family, showing association of cyst dilatation and collapse of glomeruli with some clinical features similar to MCKD/FJHN. All cysteine residues are conserved in uromodulin mammalian homologs, including C148, C150, C315 and C317 that we found mutated in MCKD/FJHN and GCKD patients. These four newly identified mutants where characterized by FACS analysis and by immunofluorescence on Hela and HEK293 after a transient transfection with wild type and mutant constructs. These experiments showed that all uromodulin mutations cause a delay in protein export to the plasma membrane (FACS analysis of Uromodulin positive HEK293 14 hours after transfection: wt = 18.65%, C149W = 5.18%, C150S = 4.65%, C315R = 9.35%, C317Y = 11.33%) due to a longer retention time in the endoplasmic reticulum as confirmed from intracellular localization by staining with antibody anti-uromodulin and antibody anti-calnexin (ER marker). Immunohistochemistry on GCKD and MCKD/FJHN kidney biopsies revealed dense intracellular accumulation of uromodulin in tubular epithelia of the thick ascending limb of Henle's loop. Electron microscopy demonstrated accumulation of dense fibrillar material within the endoplasmic reticulum. Consistently, patient urines show a severe reduction of excreted uromodulin. Further studies in families with MCKD/FJHN with new missense mutations are in progress.

Methods and Technology

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GENOTYPING OF COLORECTAL CARCINOMAS BY LASER MICRODISSECTION PRESSURE CATAPULTING (LMPC)

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Introduction: The aims of this study were to perfect the system of laser microdissection pressure catapulting (LMPC) for the assessment of the mutational status of p53 and k-ras genes. **Materials and methods:** To compare the techniques of hand- and LPC-microdissection, alcohol-fixed, paraffin-embedded tissue from consecutive series of 67 patients with colorectal carcinomas (CRC), were both hand- and laser-microdissected. In either case, dissected samples were analyzed by SSCP/sequencing and direct sequencing for k-ras and p53 gene mutations. **Results:** LMPC made (it) possible to pick up mutations by direct sequencing or SSCP/sequencing, whereas hand-microdissection mutations were identified only by means of SSCP followed by sequencing; direct sequencing did not reveal any mutationAberrantly migrating bands were found in 36% (24/67) of the cases with a total of 32 mutations. Seventy-eight percent (25/32) were found in the conserved areas of the gene; 12% (4/32) were in the L2 loop, 50% (16/32) were in the L3 loop, and 12%(4/32) in the LSH motif of the protein. Moreover, within the 67 cases examined, 40% (27/67) showed mutations in k-ras, with a total of 29 mutations identified. Of these, 14 (48%) were found in codon 12 and 15 (52%) in codon 13. **Conclusion:** This technique, used together with other modern methods of biomolecular analysis, improves the stratification of patients with CRC, thus permitting a more personalized therapeutic approach. If in the future our results are confirmed by further clinical studies involving a larger number of cases, the LMPC technique could be used in future routine clinical procedures.

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FRET: CHARACTERIZATION BY MEANS OF NANOSTRUCTURED SYSTEM ANALYSIS

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Fluorescence resonance energy transfer (FRET) is a distance-dependent physical process based on the energy transfer from a donor fluorescent molecule to an acceptor one [1]. Some crucial conditions for FRET are the following: overlap (>30%) between the emission spectrum of the donor and the absorption spectrum of the acceptor, donor quantum yield, relative orientation of the donor and acceptor transition dipoles [2]. One of the major drawbacks in the analysis of FRET-maps is due to the potential contamination by donor emission into the acceptor channel and by the excitation of acceptor molecules directly by the donor excitation wavelength (donor and acceptor spectral-bleed-through- (SBT)). Our work is focused on the refinement of FRET data to reduce SBT. Our starting point was the p-FRET algorithm recently developed at Keck Cent for Cellular Imaging, University of Virginia [3]. For this investigation, we used for the first time polyelectrolytes nanocapsules loaded with the FRET-couple FITC and Alexa594 bound to different layers at controlled distances [4]. We used a Leica SP2 AOBS spectral system and a Chameleon XR laser source.

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IMMUNOFLUORESCENCE ANALYSIS OF SAOS-2 CELLS GROWN ONTO A TITANIUM FIBER MESH SCAFFOLD

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Several porous biomaterials are used as 3D scaffolds for bone tissue engineering. Moreover, increasing evidence suggests that physical stimuli, such as shear stress and electromagnetic field, can modulate bone histogenesis and calcified matrix production *in vitro* and *in vivo* [1, 2]. Our aim was to investigate the effects of an electromagnetic wave (intensity of magnetic field, 2mT; frequency, 75 Hz) on human osteosarcoma SAOS-2 cells in terms of proliferation and calcified matrix production. SAOS-2 cells were seeded onto 3D titanium fiber mesh scaffolds, and electromagnetically stimulated ("electromagnetic culture") or not ("control culture"). The scaffolds were extensively washed with phosphate buffer saline, fixed with formaldehyde, and processed for immunofluorescence detection of some bone-specific markers, such as type I collagen, decorin, and osteopontin. Immunofluorescence analysis revealed that the electromagnetic stimulation improved the cell distribution on scaffold surface. Furthermore, the immunofluorescence analysis showed a significantly higher intensity in the "electromagnetic culture" compared to "control culture". Taken together these data seem to suggest that the electromagnetic stimulation could be used to improve osteoblast growth and calcified matrix development *in vitro*.

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A NEW TWO-STEP FLOW CYTOMETRIC METHOD TO CORRELATE APOPTOSIS AND CELL CYCLE

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The study of anticancer activity of new molecules is, actually, supported by the correlation between cellular damage events (induced by the drug) and the position of these cells along the cell cycle phases. Flow cytometry is the technique of choice to study the correlation between apoptosis and cell cycle phases. Flow cytometric (FC) techniques actually available, allowing to obtain this data, are limited by some factors. The aim of this study deals with a new cytometric procedure based on the post-fixation of Annexin V-FITC with ethanol 70%, followed by stoichiometric DNA staining with Propidium Iodide (PI) at low concentration (5 μg/ml). The method consists of two steps: I) Sample labelling with Annexin V-FITC and PI (1 μg/ml) and immediate FC analysis 1, II) Post-fixation (of the remaining part of the sample) followed by labelling with PI (5 μg/ml) and FC analysis 2. The possibility to fix Annexin V-FITC labelled cells with ethanol, was first evaluated trough fluorescence microscopy. Samples (DHDK12TRb cells, grown in serum free medium for apoptosis induction) analyzed immediately (after Annexin V-FITC) without fixation and those obtained after a three hours post-fixation treatment, were similar for specificity and intensity of FITC-labelling. Flow cytometric analyses of MOLT and Jurkat cells (treated with H_2O_2 to induce apoptosis) gave similar results. Stoichiometric PI staining after fixation allowed the dual parameter, red versus green, flow cytometry for correlated DNA/cell damage analysis of the same cell samples. Furthermore it was observed, after flow cytometric analyses of HL-60 cells (treated with the Topoisomerase-I inhibitors, Camptothecin and Topotecan), that cell cycle specificity of apoptosis obtained with this method was similar to the one obtained with the TUNEL technique applied on the same cell samples.

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CIRCULATING ENDOTHELIAL CELL SEPARATION FROM PERIPHERAL BLOOD OF PATIENTS AFFECTED BY MYELODYSPLASTIC SYNDROMES: PRELIMINARY RESULTS OBTAINED BY A NEW IMMUNOMAGNETIC PROCEDURE

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Flow cytometry (FC) had been for decades the technique of choice for studies of a large amount of cells within few minutes. More recently, FC is experimentally applied in emerging clinical applications based on the so called "rare events" detec-

tion. Beside the established problems of the monitoring of the CD4+ lymphocyte number in immunodeficient patients, other target cells are now "on focus." Very low frequency of epithelial as well as endothelial cells, circulating in the peripheral blood (PB), are "signs" of pathological development, both in solid and in haemato-oncological diseases. As far as the possibility to detect, enumerate and/or "separate" these small blood subpopulation of cells, other than FC, the immunomagnetic separation technique is actually a valid alternative (and/or a complementary) approach. In order to contribute to this methodological area we developed and tested a "microchamber" magnetic device aimed to the detection and counting of lymphocyte subsets (CD14, CD4, etc) in few ml of whole blood. Further development of this technical approach is now dedicated to the "rare events" capture and separation in a large volume of sample by means of a "macro-chamber" able to treat up to 10 ml of blood. The system has been tested for the detection and separation of endothelial cells in the peripheral blood of patients suffering from myelodysplastic syndromes. Buffy-coat from 10 ml of PB has been labelled with 20 ml of PE-conjugated mouse anti-human CD146 (Pharmingen, Becton Dickinson) and after two washings, with 50 ml of magnetic beads coated with a monoclonal anti-mouse Ig (Pan Mouse IgG Dynabeads, Dynal Biotech). The first reaction has been checked by fluorescence and the second by bright field microscopy. Cell suspension (unlysed) has been deposed in the "macro-chamber" faced on the magnet and after 1min rosetted-magnetically-captured cells have been washed with repeated steps in PBS (20 ml). Non-rosetted non-attracted cells, free to float during washings, were discarded while the rosetted ones are blocked at the well bottom. After magnet removal this cell sub-fraction can be recovered for any additional cytochemical and/or molecular investigations.

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MAGNETO-MECHANICAL ADHERENT CELL DETACHMENT: ADVANTAGES AND LIMITS AS COMPARED TO ENZYMATIC TREATMENTS

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Cells growing adherent in flasks or other plastic containers, generally require to be detached and recovered as a cell suspension to be re-seeded and/or be examined by both microscopy and flow cytometry. Cell detachment is actually largely performed by means of enzymatic treatments, generally based on the use of trypsin. This enzyme is able to digest the cell surroundings and, after a proper controlled treatment, cells can be induced to float in the medium thanks to a few mechanical shakes. The enzymatic action, even if perfectly controlled and inhibited (at the end of the process), causes a consistent cell loss and, depending on the type of cell culture, a great amount of debris is also produced. The cell suspensions obtained by this detaching method shows a good morphology even if viability, as checked by Propidium Iodide, may be in the order of 60–80%, according to the cell type and accuracy of the applied procedure. Alternative methods to overcome the use of enzymatic treatment have been proposed and are essentially based on the use of chemical agents, able to chelate the calcium ions thus yielding a release of cell-to-cell bindings and therefore a simplified cell release by mechanical shake of the flask. Finally, traditional mechanical methods based on the use of different shaped "scrapers" have been proposed even in combination with the previous chemical-enzymatical aids. A new magneto-mechanical procedure has been developed, based on the concept to automate different "magnetic particles" acting as micro-scrapers driven by an external magnetic field. A continuously variable magnetic field generator (Consul TS, Turin-Italy) has been used in combination with different magnetic particles coated by chemical inert resins (Akzo Nobel, Como-Italy). Preliminary data obtained on a cell line DHDK12TRb and compared with the trypsin method allowed to state that: 1) The magneto-mechanical procedure is more rapid, simple, and reproducible; 2) the total cell yield is consistently higher and with significant less debris; 3) the cell viability (as PI exclusion) is significantly lower. This last finding is still under investigation because PI+ cells are not all definitely dead cells, being able to proliferate proliferate when re-seeded in fresh medium.

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FLOW CYTOMETRIC AND IMMUNOFLUORESCENCE ANALYSIS OF *STREPTOCOCCUS AGALACTIAE* 6313, A FIBRINOGEN-BINDING STRAIN

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Binding of *Streptococcus agalactiae*, the causative agent of meningitis in neonates, is mediated by a new discovered adhesin designated FbsA [1]. The *fbsA* gene encodes a protein that possesses structural similarity to many cell surface-associated proteins from Gram-positive bacteria. In addition, FbsA contains a domain composed of repetitive units, each 16 amino acids in length, located just outside the cell wall attachment region and involved in the adherence of streptococci to human fibrinogen. In the present study we describe the generation and characterization of a mouse monoclonal antibody, 5H2, raised against a synthetic peptide of FbsA corresponding to the repeat unit of FbsA and its use to detect the expression of the protein on the surface of streptococcal cells by THROUGH flow cytometric and immunofluorescence analysis. Furthermore, we investigated the inhibitory effect of isoforms of FbsA containing a variable number of repetitive units on the binding of FITC-conjugated fibrinogen to *S. agalactiae* cells.

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PHOTOACTIVATION OF PAGFP BY MEANS OF OPTICALLY CONFINED NON LINEAR METHODS

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This work reports about the two-photon activation and excitation properties of the PA-GFP, a photo-activatable variant of the Aequorea Victoria green fluorescent protein (1) in the spectral region from 720 to 920 nm (2). It is known from this special form of the molecule, that it has an increased level of fluorescence emission when excited at 488 nm following irradiation at ~413 nm, under singlephoton excitation conditions (1). Here, we show that upon two-photon irradiation, PA-GFP yields activation in the spectral region from 720 to 840 nm. Following photoactivation, the excitation spectrum shifts maintaining the same emission spectrum of the single-photon case for the native and photoactivated protein. We used a Leica SP2 AOBS spectral system and a Chameleon XR laser source. Two-photon activation allows to get a better optical confinement of protein activation when compared with the conventional activation. In order to evaluate other confinement modalities we photoactivated paGFP under Total Internal Reflection Fluorescence (TIRF) conditions (3). A comparison betwteen two-photon and TIRF photoactivation on living cells applications is reported.

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FLOW CYTOMETRIC ANALYSIS OF A MONOCLONAL ANTIBODY AGAINST ClfB, A STAPHYLOCOCCAL FIBRINOGEN BINDING PROTEIN

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Staphylococcus aureus is an important pathogen that causes a significant number of community-acquired and nosocomial infections worldwide. The clumping factor B (ClfB) of *Staphylococcus aureus* is a surface protein that binds to fibrinogen and cytokeratin 10 [1,2]. ClfB was shown to be a critical virulence factor in several experimental models of infections. In this study we describe the generation and characterization of a murine monoclonal antibody, 3D6, against region A of ClfB, which is composed of three subdomains named N1, N2 and N3. 3D6 recognizes in an ELISA assay the full-length region and the combination of motifs N2N3, but not the individual subdomains, suggesting that the target of the antibody is a conformational epitope located at the interface between N2 and N3 subdomains. The epitope was also recognized by flow cytometric and immunofluorescence analysis on the surface of *Lactococcus lactis* cells transformed with the vector *pKS80* harbouring the *clfB* gene. It is discussed the possibility that 3D6 might be used as a probe to detect the expression of ClfB on the surface of clinical isolates of *S. aureus*.

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Microscopy

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NEW HORIZONS IN FLUORESCENCE MICROSCOPY IMPROVED BY LIGHT EMITTING DIODES (LEDs)

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Light Emitting Diodes (LEDs) technology greatly improved in these last few years. From small lighting indicators of a few milliWatts, now LEDs are also available in the range of some Watts of power, making them attractive to replace arc lamp excitation sources for both fluorescence microscopy and flow cytometry. Different prototypes of excitation modules, fitting most of commercially available microscopes and operated by both epiillumination and transmitted light, have been constructed and tested. Instrumental and "visual" comparisons between LEDs and lamp excitations have been performed. Instrumental measurements have been carried out with a power meter located under the micro-

scope objectives, while the visual comparison have been performed by means of various biological samples labelled with several fluorochromes. The results obtained indicates that the excitation performances of 3 Watts LEDs and 100 W mercury arc lamp are comparable. A significant increase of signal-to-noise ratio is achieved particularly with an original "transmitted excitation" set up, by means of both 2 W UV (365 nm) and 3 W Blue (485 nm) LEDs, delivering up to some hundreds milliWatts within narrow spectral bands (15 nm). Compared with the standard lamp excitation, FITC labelling observation is consistently improved by blueLED excitation, whereas the optical power emitted by a mercury arc lamp in this spectral region is not very high. Our data indicates that LEDs technology applied to fluorescence microscopy allows improvements both in term of image quality and system efficiency.

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SHG MICROSCOPY APPLIED TO THE STUDY OF BONE FRAGMENTS

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The goal of this communication is to report about a recent study on bone fragments obtained from biopsies of human patients of different ages and sex by means of three-dimensional microscopy. Samples were selected from 8 patients and classified according to age into subgroups. Confocal and two-photon excitation microscopes allow to get three-dimensional information from thick samples like the ones we are dealing with bone fragments. Second harmonic generation (SHG) microscopy is coming into use as a tool for studying the distribution of collagen within the 700–1100 nm range. It is promising in characterizing collagen. We coupled SHG with confocal and two-photon excitation imaging. Morphological and functional properties of such bone fragments can be studied by exploiting auto-fluorescence, mainly due to collagen content. For our studies it was mandatory the use of a scanning head endowed of spectral capability. Moreover, for both two-photon excitation and SHG imaging we needed a tunable ultrafast laser source. We used a Leica SP2 AOBS spectral system and a Chameleon XR laser source. Two-photon excitation fluorescence was collected in a de-scanned mode while SHG was captured both in the forward and in the backscattering direction. We are also considering the role of advanced glycation endproducts (AGEs) formed in long-lived matrix proteins by a non-enzymatic reaction with sugar. We observed an increase of autoflorescence in those bone fragments belonging to the old subgroup compared to the autofluorescence of young patients. So far, we speculated that this effect could be due to the increasing concentration of the AGEs products that must be higher in the osteoporotic subject.

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GLIA RE-SEALED PARTICLES FRESHLY PREPARED FROM ADULT RAT BRAIN ARE COMPETENT FOR EXOCYTOTIC RELEASE OF GLUTAMATE

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Glial sub-cellular particles (gliosomes) were purified from rat cerebral cortex and investigated for their ability to release glutamate. Confocal microscopy showed that the glial specific proteins GFAP and S-100β, but not the neuronal proteins PSD-95, MAP-2, and β-tubulin-III, were enriched in purified gliosomes. Nearly 90% of the organelles present in the preparation were GFAP or S-100β positive. Ultrastructural analysis highlighted morphological differences between gliosomal and synaptosomal preparations. The Ca^{2+} ionophore ionomycin (0.1–5 μM) stimulated the release of [$_3$H]D-aspartate and endogenous glutamate. Gliosomal glutamate release was dependent on $[Ca^{2+}]_o$ and sensitive to bafilomycin-A1, suggesting the involvement of exocytotic processes. Accordingly, ionomycin induced a Ca^{2+}-dependent increase of the vesicular fusion rate, monitored with acridine orange. Also KCl (15, 35 mM), 4-aminopyridine (0.1, 1 mM) or veratrine (1, 10 μM) induced a Ca^{2+}-dependent [$_3$H]D-aspartate release from gliosomes. KCl increased both membrane potential and $[Ca^{2+}]_i$, measured by rodhamine-6G and FURA2, respectively. This increase was dependent on $[Ca^{2+}]_o$ and sensitive to Cd^{2+}. Confocal microscopy revealed that the gliosomal fraction contains proteins of the exocytotic machinery (syntaxin-1, VAMP-2, SNAP-23, SNAP-25) co-existing with GFAP immunoreactivity. Moreover, GFAP or VAMP-2 were co-expressed with the vesicular glutamate transporter. Ultrastructural analysis showed that several ~30 nm non-clustered vesicles are present in gliosome cytoplasm. In conclusion, the present results suggest that purified gliosomes contain glutamate-accumulating vesicles and that they can release the amino acid by a process resembling neuronal exocytosis. Supported by grants from Italian Ministry of of Univ. and Scient. Res. (COFIN-MIUR 2002, 2004 and FIRB 2003).

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EVALUATION OF BACTERIAL ADHESION TO PEG MODIFIED TITANIUM OXIDE SURFACES BY FLUORESCENCE MICROSCOPY

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Today, more than ever, titanium is considered the preferred biomaterial for fabricating load-carrying implants for bone applica-

tions, such as hip joint replacement and dental implants, due to its mechanical properties similar to bone, low specific weight, corrosion resistance properties [1]. Current research is focus on the improving titanium *in vivo* performance, by means of the design of the interface between implant and biological *medium*: the titanium oxide surface. In fact, although the clinical use of titanium-based dental implant is common and has produced high success, in some cases, implant failures are still reported, principally due to osteointegration loss and periimplant bacterial adhesion. In view of the tested poly(ethylene glycol) (PEG) properties of reducing bacterial adhesion [2], titanium oxide (TiO_2) surfaces were modified by PEG-terminated alkane phosphate self assembly monolayers (SAMs). In order to test the bacterial adhesion dependency on PEG surface density, mixed SAMs were prepared by mixing equimolar solutions of PEG terminated alkane phosphate (PEGmal-S-UDPO$_4$) and 12hydroxydodecyl phosphate (OH-DDPO$_4$). The PEG modified surfaces were incubated (2 h) with a cariogenic oral bacterial strain (S.mutans) and then observed by means of fluorescence microscope. Bacterial adhesion, quantified by means of fluorescence microscope images processing, was demonstrated to be reduced on PEG modified surfaces up to two orders of magnitude respect to the uncoated surfaces. These results are extremely promising in view of a potential clinical application in dental implants field, where avoiding the bacterial adhesion is highly desirable.

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PROTECTION CAPABILITY OF POLYELECTROLYTE LAYERS: A SACCHAROMYCES/PARAMECIUM MODEL

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The development of an encapsulation method for biological cells endowed of sufficient permeability that oxygen and nutrients can feed the cells, and appropriate cellular products can be released in a programmed way, can open new avenues in the realization of artificial cellular networks and smart biosensors [1]. The capsular material should be restrictive enough to prevent encapsulated cells from the attack of specific proteins and enzymes filtered by the permeability properties of the capsule itself. A suitable system to this end can be designed and realized using charged polyelectrolytes assembled by means of the Layer-by-Layertechnique [2, 3]. In the present work we assess the ability of polyelectrolyte nanostructured shells to protect *Saccharomyces cerevisae* yeast cells inserted within a heterologous organism, the protozoan *Paramecium primaurelia*, against lysosomial enzymes attack. To investigate the influence of preparation conditions we used PE solutions at three different ionic strengths. This allowed to stress the role of salt concentration on the properties of the multilayer shell. As well, we modulated the number of layers of the shell in order to derive those conditions enabling protection of the cells from digestion by *Paramecium's* lysosomal enzymes. Yeast cells viability was tested by observing population growth dynamics. This study has been carried out by means of confocal laser scanning microscopy and fluorescence labelling [4]. Results about optimal conditions for the protection of the yeast against destruction by lytic enzymes are reported [5].

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THE ISOFORMS ERK 1 AND 2 HAVE DIFFERENT CAPACITY OF PHOSPHORYLATING NUCLEAR TARGETS BECAUSE OF DIFFERENT MOBILITY THROUGH THE NUCLEAR MEMBRANE

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The kinase ERK1/2 is a crucial effector linking extracellular stimuli to long term cellular responses. Upon stimulation ERK1/2 translocates to the nucleus leading to the activation of specific programs of gene expression. Recently it has been demonstrated that the two isoforms of ERK, ERK 1 and 2, exert different effects: while the genetic ablation of ERK 2 is lethal, the loss of ERK 1 causes an upregulation of ERK 2 leading to altered plasticity and behaviour. ERK 1 and 2 are very similar, mainly differing because of the presence of a short sequence of about 20 residues at the N-terminal of ERK 1: since no specific functional domain is mapped here, there is no explanation for the different actions of the two molecules. We speculated that these differences might depend on the processes controlling activation and nuclear translocation of ERK. To this effect we visualized the dynamic of ERK localisation by transfecting fluorescently tagged ERK 1 and 2. Dynamic imaging demonstrated that nuclear localization depended on the equilibrium between cytosolic activation and rapid nuclear dephosphorylation. Therefore, the pool of phosphorylated ERK in the nucleus is maintained by a continuous shuttling through the nuclear membrane. We found that a fast shuttling rate is necessary to compensate the rapid nuclear dephosphorylation. ERK 2 shuttles through the nuclear membrane about 4 times faster than ERK 1 and it is therefore far more capable than ERK 1 to support the phosphorylation of downstream targets. These results show that nucleocytoplasmic trafficking and mobility of ERK 1 and 2 are dynamically regulated and that it is a crucial determinant of the capability of the pathway to convert extracellular stimuli in long lasting changes of neuronal function.

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FRAP ON NANOSTRUCTURED MODEL SYSTEMS: EVALUATION OF TIME DEPENDENT ARTEFACTS

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Fluorescence Recovery After Photobleaching (FRAP) is a fluorescence technique widely used to identify and quantify the diffusion of fluorescent labelled molecules in biological samples with an optical microscope.[1] In a typical FRAP experiment, the fluorophores in a chosen region are irreversibly "turned off" by photobleaching. Then the redistribution of the fluorescence, due to the mobility of the marked molecules, is monitored and the fluorescent signal inside the region increases until an equilibrium is reached. By analysing the curve of fluorescence recovery with a suitable theoretical model, the kinetic proprieties of the labelled molecule can be evaluated. However, even in the simpler case of determination of free diffusion coefficient via FRAP analysis this kind of quantitative interpretation can lead to wrong results due to many aspects that are not directly taken in account by the model used. First of all, many of the algorithms neglect diffusion processes during the bleach time. If this effect is not explicitly taken into account by the theoretical algorithm, the evaluated diffusion coefficient will be underestimated[2] ("corona effect"). In the present study we are investigating the influence of the "corona effect" on the evaluation of diffusion events in heterogeneous samples. As model system, micrometer sized cubic polyelectrolyte capsules[3] were used. The diffusion coefficients for dextran molecules of different molecular weight were determined for the passage through the multilayer polyelectrolyte matrix. As a result this work shows that an empirical approach works properly to determine the influence of the "corona effect" also for heterogeneous systems. We used a Leica SP2 AOBS spectral system.

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IMPROVEMENT OF RESOLUTION IN THREE-DIMENSIONAL FLUORESCENCE MICROSCOPY THROUGH THE WEB WITH "POWER-UP YOUR MICROSCOPE"

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The fluorescence microscope can image a specimen in its natural environment forming a 3D image of the whole structure allowing in vivo and in vitro observations. However, the image formation process is affected by distortions mainly due to blurring and noise. These distortions hide fine details in the image hampering both the visual and the quantitative analysis. From a mathematical point of view the properties of an image formation system, based on the optical microscope, can be modelled by the knowledge of the Point Spread Function (PSF), i.e., the image of a point-like source of subresolution dimension approximating impulse response of the system. Under some assumptions and in the absence of noise, the registered data are due to the convolution of the original object intensive properties with the PSF. "Power-Up Your Microscope"[1] implements different deconvolution algorithms to compute the best approximation of the original object. This is possible by uploading own images and compiling a form with few acquisition microscope parameters. The software package finds automatically the theoretically PSF, which will be used for the image restoration process. Image deconvolution can be performed on images coming from wide field, confocal and multiphoton microscopy. In order to compare the effective improvement in the quantitative image formation analysis, we compared the z-response of the optical system before and after restoration by using polyelectrolyte self-assembled ultra-thin (2–2.5 nm) layers transferred onto a coverslip or used for fabrication of round shaped shells. Such a sample allows to test the performances of the different computational approaches implemented in the web-based package.

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THE OXIDATION PRODUCTS OF SPERMINE INDUCE CYTOTOXIC EFFECTS ON CULTURED TUMOR CELLS WITH MULTIDRUG RESISTANT PHENOTYPE

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The occurrence of resistance to cytotoxic agents in tumor cells is one of the major obstacle to successful anticancer chemotherapy. Multidrug resistance (MDR) is often associated with the overexpression of drug transporters, such as P-glycoprotein (Pgp), which acts as a drug efflux pumps. The activity of these proteins produces changes in intracellular drug concentration and distribution. We have characterized through flow cytometry and confocal microscopy two pairs of sensitive and resistant human tumor cell lines (colon carcinoma LoVo and melanoma M14) for the expression of drug transporters and doxorubicin (DOX) accumulation and distribution. Then, using bovine serum amine oxidase (BSAO), that catalyses the oxidative deamination of polyamines with the formation

of hydrogen peroxide (H_2O_2) and aldehydes, we verified whether the oxidation of spermine had a cytotoxic effect on tumor cells and, particularly, on MDR cells. Surprisingly, the evaluation of cell survival showed that both types of MDR cells were more sensitive than their wild type counterparts. E. M. observations showed mitochondrial modifications more pronounced in MDR cells than in sensitive ones. To analyze the mitochondrial functionality, a flow cytometric study was performed on cells treated with BSAO + spermine or with exogenous H_2O_2. The mitochondrial mass was evaluated after labeling with the fluorescent probe NAO while the dye JC-1 was used to detect the mitochondrial membrane potential modifications. The results obtained demonstrated that the higher cytotoxic effect could be ascribed to a basal hyperpolarized status of the mitochondria in resistant cells with a consequent evident membrane depolarization. These results suggest promising applications of BSAO in anticancer therapy, mainly against MDR tumors.

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FLOW CYTOMETRIC DETECTION OF BONE MARROW INVOLVEMENT IN RHABOMYOSARCOMA PATIENTS

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Rhabdomyosarcoma (RMS) is a malignant tumor of mesenchymal origin, thought to arise from cells committed to a skeletal muscle lineage, and is the most common soft-tissue sarcoma of childhood. The presence of metastatic disease at diagnosis, particularly in the bone (BM), significantly correlates with adverse prognosis. To identify RMS BM infiltrating cells, we evaluated the CD45−/CD56+ phenotype by flow cytometric (FC) assay. The lowest sensitivity threshold of the assay was established at 0.01% by spiking experiment. RMS cells were detected at diagnosis in the BM of 4 out of 24 patients (16%) affected by RMS admitted at our institute. Furthermore, RMS BM cells were detected at relapse in 2 of these 4 patients. Interestingly, 1 of these patients displayed 0.01% of CD45−/CD56+ cells and the detection was missed using standard morphological methods. In order to validate the CD45/CD56 assay, we correlated the FC results with reverse transcriptase polymerase reaction (RT-PCR) for miogenin (Myf4). We were able to detect 10^2 TE 671 added to 10^6 normal cells. No discrepancy between molecular analysis and FC assays was revealed. We detected a strong FC signal for CD90 in RMS TE671 cell line. For this reason, the CD90 were added to CD45/CD56 and the results are currently under investigation. FC immunophenotyping is underused as a diagnostic tool for non-hematopoietic neoplasms; however, our results indicate that FC has a potential application in BM assessment of RMS patients.

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GENERATION OF CYTOMEGALOVIRUS (CMV)-SPECIFIC CD4 AND CD8 T CELL LINES USING PROTEIN-SPANNING POOLS OF pp65 AND IE1 DERIVED PEPTIDES

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Reactivation of latent CMV in immunocompromised recipients of allogeneic stem cell transplantation remains a major cause of morbidity and mortality. Reconstitution of immunity by CMV specific immunotherapy is an attractive alternative to drugs currently used, which show high toxicity and are sometimes ineffective. It has been demonstrated that CD4 helper T-cell function is crucial for the persistence of *in vivo* transferred CD8 CMV-specific CTL. Based on this finding, we have explored the feasibility of generating both anti-CMV CD4 and anti-CMV CD8 T-cell lines. Dendritic Cells (DC) were generated from donor monocytes after a 7-day culture in the presence of GM-CSF plus IL-4 and matured with TNFα, IFNα, IFNγ, IL1β, POLI I:C. Matured-DC were then pulsed with a pool of 50 peptides spanning pp65 and IE1 proteins which are recognized by both CD4 and CD8 T lymphocytes. Donor T cells were stimulated three times on day 0, +7 and +14 with maturepeptide pulsed-DC. Cultured T cells expressed CD8 (mean = 70%, range 60–81%) and CD4 (mean = 20%, range 15–28%) and showed a CD45RA-CCR7− Effector Memory (mean = 26%, range 19–30%) or a CD45RA+CCR7− Effector Memory RA+ phenothype (mean = 67%, range 59–77%). Furthermore, 90% of CD8+ and 40% of CD4+T cells expressed high levels of perforin and granzyme. An enriched CMV-specific T cell population was observed after staining with pen-tamers (7–45% pentamer+ T cells). In 4/5 cases tested, cultured T cells showed a cytolitic activity against CD8 peptide pulsed target cells (average lysis = 50% at E:T ratio of 30:1, range 40–55%) and to a lesser extent against CD4-peptide pulsed target cells (average lysis = 35% at E:T ratio of 30:1, range 30–40%). In addition, cultured T cells were able to proliferate and to produce IFNγ after exposure to peptide-pulsed DC. In conclusion, a great advantage of this method is represented by the possibility to generate anti-CMV CD4+T cells, which could support *in vivo* the persistence of re-infused CMV-specific CTL. Moreover, the possibility of generating peptides under GMP conditions would facilitate the translation of this approach into clinical intervention.

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AUTOLOGOUS MESENCHYMAL STEM CELLS: CLINICAL APPLICATION IN AMYOTROPHIC LATERAL SCLEROSIS

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Background: Mesenchymal Stem Cells (MSCs) from bone marrow (BM) are characterized by high plasticity. They

can differentiate into multiple mesenchymal lineages and into neuron-like cells suggesting that MSCs may overcome germ layer commitment. MSC transplantation represents an attractive and promising approach in neurodegenerative diseases such as ALS, a motor neuron disease leading to a linear decline in muscular function, in paralysis and dysphagia. Within 2 to 5 years of clinical onset death, due to respiratory failure, occurs. There are no therapies available today, and new therapeutic strategies might be identified. **Objectives:** Our study was aimed to evaluate the feasibility and safety of intraspinal cord implantation of autologous MSCs in ALS patients. **Methods:** Nine patients affected by definite ALS were enrolled, monitored for 6 months before MSC implantation and followed for at least 3 years. MSCs were isolated from BM, expanded in vitro for 4 weeks and suspended in 1 ml of autologous cerebrospinal fluid before implantation into the spinal cord. **Results:** No patient manifested major adverse events such as respiratory failure or death. Minor adverse events were intercostal pain irradiation and leg sensory dysesthesia, both reversible after a mean period of 6 weeks. No modification of the spinal cord volume or other signs of abnormal cell proliferation were observed. Two patients died for the disease progression respectively 9 and 24 months after MSCs implantation. A significant slowing down of the linear decline of the forced vital capacity and of the Functional Rating Scale (ALS-FRS) were evident in 4 patients while in 3 patients no modification of the natural course of the disease was observed 36 months after transplantation. **Conclusions:** Our results demonstrate that direct injection of autologous expanded MSCs into the spinal cord of ALS patients is safe, with no toxicity, and well tolerated. The clinical results seem to be encouraging.

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PHERIPHERAL BLOOD AND EX VIVO-GENERATED DENDRITIC CELL CHARACTERIZATION BY FLOW CYTOMETRY ANALYSIS IN CANCER PATIENTS TREATED WITH A NEW CELL THERAPY APPROACH

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Extracorporeal photoapheresis (ECP) is able to provoke monocyte (Mo) to DC differentation and tumor cell apoptosis. In 4 cancer patients (pts), Mos were collected by leukapheresis, activated by ECP with 8-methoxypsoralen and left overnight in co-colture with antigen tumor lysate, before being reinfused in patients (pts). Ex vivo DC generation was also performed starting from activeted Mo. Circulating DCs were identified as mature DCs CD83+, myeloid DCs (mDCs) HLA-DR+ BDCA1+ and plasmacytoid DCs (pDCs) CD123 high+BDCA2+. They were evaluated as % increase after overnight treatment. Ex vivo DCs were generated from Mo following the standard procedure. At day + 7, non maturated DCs were analyzed for HLA-DR, CD14, CD1a, CD83 and CD86 expression. They were evaluated before treatment (t0) and at cycle 5 (t1) and 15 (t2). No difference in the % increase of mature DCs CD83+ after overnight treatment was showed in 3 pts; in one of them a significantly decrease was observed (median % decrease: 49.9, range: 33.3–66.6); mDCs were decreased in 3 pts (median % decrease: 36.1, range: 0–57.1); one resulted increased (median % increase: 150, range 0–300); a significantly % increase was observed for pDCs in the 2 pts with better clinical response (CR) (median % increase: 39.25, range: 20–61) and no difference in the 2 with worse CR. In both pts with better CR, the % of ex-vivo generated DCs significantly increased at t2 compared with t0 (30 vs 75 and 27 vs 43, respectively); in contrast, no increase was observed in the pts having worse CR (27 vs 30 and 70 vs 40, respectively). In all pts, DCs showed similar % of HLA-DR expression at each time point, a slightly increase of CD83 at t1 and a decrease in the CD86 expression. No expression of CD1a and CD14 was evidenced. These results indicate that: 1) mature DCs are present after pulsing with autologous tumor, and they are increased in their plasmacytoid subset and 2) DCs, showing a mature phenotype, can easily be generated from these pts.

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FLOW CYTOMETRIC DNA CONTENT ANALYSIS IN 708 PATIENTS AFFECTED BY OVARIAN CANCER. CLINICAL CORRELATIONS

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The flow cytometric DNA Index has been evaluated in 822 samples like primary tumor, metastases and ascitic fluids, obtained from 708 patients affected by ovarian cancer at different clinical FIGO stage: 175 FIGO I, 38 FIGO II, 500 FIGO III and 78 FIGO IV. 292 tumors had a DNA diploid content while the other 530 had a DNA aneuploid content. In 95 patients the DNA index was measured on several samples and, over time, in the same patient showing that the DNA index was very stable. Tumor FIGO stage and ploidy was significantly associated: in patients with tumor FIGO stage I and II, 115 had tumors with diploid DNA content and 98 with aneuploid DNA content, while stages III and IV were more likely to be DNA aneuploid, being 153 with diploid DNA content and 425 with aneuploid DNA content ($p < 0.01$). The DNA index was also related to the degree of differentiation of the tumors: poorly differentiated tumors were more likely to be DNA aneuploid tumors ($p < 0.01$). A strong association was found between ploidy and residual tumor size at first surgery : patients with residual tumor size >2 cm had a significantly large number of DNA aneuploid than DNA diploid tumors ($p < 0.01$). The percentage of the cells in the S phase of the cell cycle evaluated in tumors at FIGO stage III and IV was significantly higher in DNA aneu-

ploid and in poorly differentiated tumors than DNA diploid and well differentiated tumors. Multivariate analysis using Cox model performed in borderline tumors, or in tumors at FIGO I and II, or in tumors at FIGO III and IV clinical stage showed that the DNA index was not considered as an independent prognostic factor in this study. In patients with FIGO III and IV tumors the residual tumor size and histological mucinous and clear cells tumor type were found to be an independent prognostic factors.

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FLOW CYTOMETRY AND FLUORESCENCE MICROSCOPY CHARACTERIZATION OF DISSOCIATED HEPATIC TISSUE FROM ORGAN DONORS

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There is a growing interest on the immunophenotypical characterization of dissociated hepatic tissue. Hepatic cells can be used for therapeutic purposes (cell transplantation, bioartificial liver devices), and for studies on liver biology and disease pathogenesis. In this study we defined the antigenic profile of parenchymal and stem cells contained in the liver of organ donors. We performed four–colors flow cytometry (FC) evaluations of liver mononuclear cell suspensions (L-MNC) obtained after enzymatic digestion of liver biopsy specimens (n = 7, 20–0,045 gr). L-MNC were incubated with the following antibodies: antiCD34, -CD133, -CD45, -CD117 (c-kit), -CXCR4, -CD29, CD49f, -CD105. Cell viability was assessed using 7-AAD. Fluorescence microscopy (FM) was performed on tissue criosections to evaluate the expression of either surface markers (see FC) and ASGPr, or cytoplasmatic proteins, alpha-fetoprotein, albumin and cytokeratin 7 and 18. The FC results, expressed as mean and standard deviation, are summarized in the Table.

mean SD

		$CD34_+$				
			$CD45_+$		CD45	
viability	$CD45_+$			c-kit_+		c-kit_+
79,14	20,59	0,45	78,14	50,71	21,86	10,24
15,85	9,13	0,37	27,74	18,62	27,74	22,44

In the compartment of the CD34+/CD45+ cells we were able to identify a subset of cells expressing both CD133 and CXCR4, while they were undetectable in the liver parenchymal compartment. The CD49f and CD29 antigens were expressed on the majority (90%) of intrahepatic CD34+ cells, while CD105 antigen was present in 70%. These observations were also confirmed by FM. The combination of several stem and hepatic markers gives a dynamic description of liver cell subpopulations. FC analysis of $CD34_+$ stem cells allows the identification of at least two phenotipically distinct populations. The first one co-expressing CD45 resembles the classical hematopoietic stem cell and harbors CD133 and the chemokine receptor CXCR4 for trafficking to the liver in response to liver injury. The second population is increased in the intrahepatic fraction and showed the typical stem cell tissue marker c-kit.

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ROLE OF CXCR4 IN MELANOMA: A PROGNOSTIC FACTOR AND A NEW TARGET OF THERAPY

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Accumulating evidences focused on the role of chemokines and chemokines recepors in spreading of cancer cells. Chemokines are a family of small, 8-10 kDa inducible and soluble proteins. The mechanism of chemokine action involves initial binding to specific seven transmembrane spanning G protein-linked receptors on target cells. Chemokines are involved in chemotaxis, angiogenesis and hematopoiesis. CXCL12, a member of the chemokine family, produced by the lymph node, lung, liver, and bone marrow is a potent chemoattractant for T lymphocytes, neutrophils and CD34+ hematopoietic progenitors. CXCR4 is the unique receptor of CXCL12 and is expressed in many different tumors. A prognostic role for CXCR4 expression was recently demonstrated in human primary melanoma. We hypothesized that the CXCL12-CXCR4 biological axis is important in mediating human melanoma metastasis. Metastatic melanoma cell lines expressed different levels of CXCR4; PES43, Alo40 and COPA cell lines showed the highest levels of the CXCR4 (>90% of positive cells), while PES 41, Alo39, PES47, POAG and CIMA cell lines showed low-moderate degrees of expression (5–65% of positive cells) and CXCL12 expression was detected in PES 47. Human melanoma metastasis cell lines undergo chemotaxis in response to CXCL12. CXCL12-CXCR4 activation showed mitogen-activated protein kinase activation with enhanced extracellular- signal- related kinase1-2 without change in apoptosis. The inhibition of Erk 1-2 activation with AMD3100 treatment confirmed the specific CXCL12 induction. Moreover CXCL12 induced cellular proliferation in PES41, PES43 and PES47 cells. In vivo experiments showed that PES43 human melanoma cells were tumorigenic in nude mice and the induced tumor expressed CXCR4. Taken together these evidences strongly support the role of CXCR4 inhibition in melanoma patients wishing active inhibitors such as AMD3100 to experience in human melanoma clinical trials.

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CYTOFLUORIMETRIC DETECTION OF DNA PLOIDY: A POSSIBLE PROGNOSTIC MARKER IN ENDOMETRIAL CARCINOMA

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The assessment of cell ploidy has been consistently suggested as an useful prognostic marker in gynecological cancers. Literature data, indeed, indicate that 15% of stage I endometrial carcinomas are aneuploid, with a referred incidence of aneuploidy rising with the increasing of the performed studies. Moreover, the occurrence of a better prognosis for patients with diploid tumors, has been consistently referred, while the mitotic index has been observed to behave as an independent prognostic indicator. In addition, the relevance of flow cytometry techniques to directly investigate the ploidy status in fresh and/or paraffin embedded tumor specimens has been largely recognized. In order to optimize the clinical management of endometrial carcinoma this study report the analysis, by flow cytometry, of DNA ploidy assessment in a cohort of 89 patients with 48 months of follow-up. In our patient cohort 35% of G3 endometrial carcinomas have been observed to be aneuploid, with 36% of aneuploid tumor in the serous papillar type and 16% in the clear cell variants. A possible correlation between the clinical outcome and the analysis of DNA ploidy, as an independent prognostic marker for endometrial adenocarcinoma, will be investigated.

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HUMAN PULP STEM CELLS ARE ABLE TO DIFFERENTIATE IN VITRO INTO BONE-LIKE PRODUCING OSTEOBLASTS

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Recently it has been demonstrated that dental pulp stem cells from human teeth are able to produce in vitro sporadic but densely calcified nodules and to form mineralized tissue after transplantation in vivo. This study is characterizing a new population of stem cells derived from human exfoliated deciduous teeth as well as from permanent teeth (all VIII molars) obtained from 19 to 37 year old individuals. This new cell population, selected by FACSorting, is c-kit+, CD34+, STRO-1+ and CD45− and spontaneously differentiates into CD44+, CD54+, RUNX2+ osteoblasts able to produce an extracellular inorganic matrix that mineralizes becoming fibrous bone, in vitro. The mineralized bone tissue is positive for ALP, Schmorl and alizarin red reagents while the osteoblasts are positive for osteonectin, osteopontin, fibronectin, collagen III and BAP, strongly resembling the human mesenchymal tissue during mineralization. These data suggest that a dental pulp stem cell population (c-kit+/CD34+/CD45−), which we call Stromal Bone-like-Producing SBP, is capable to differentiate toward several stromal-derived differentiated cells and mainly generates self-maintaining and renewing osteoblasts. These cells have been observed to continuously produce a hard bone-like tissue, strongly resembling the human bone tissue during mineralization. The possibility that such tissue could represent an ideal source of osteoblasts and mineralized tissue for bone regeneration (i.e. tissue-based clinical therapies) and transplantation might also be investigated.

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IMPACT OF DOSE–DENSE CHEMOTHERAPY ON THE IMMUNE SYSTEM OF EARLYS BREAST CANCER (EBC) PATIENTS

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Recent clinical trials suggest that Anthracycline (A)/Taxane (T)-based, dose-dense chemotherapy (CT) may improve the survival, compared with a conventional schedule for EBC pts. Peg-Filgrastim (Peg-FIL) is a recently developed cytokine utilized to reduce the incidence of febrile neutropenia in pts receiving myelosoppressive CT as well as to both increasing the CT dose and shortening the intervals of CT. To date no information are available regarding the possible impact of a dose-dense CT with Peg-FIL support on the immune system of EBC. 11 EBC pts were treated with an AT-based, dose-dense CT program with Peg-FIL support, administered 72 h after each CT course. The phenotype of peripheral blood lymphocyte(PBL) and the circulating dendritic cell (DCs) pattern, were determined before starting treatment, immediately before each CT course and one months after the discontinuation of CT. Before starting CT, the absolute leucocytes and T lymphocytes (CD4+ and CD8+), B lymphocytes (CD19+ and CD20+), NK(CD16+ and CD56+) counts and DCs were not significantly altered in CT-naïve pts with respect to 20 ND utilized as control. During CT, the CD19+, CD20+, CD20+/CD38+ subset were statistically decreased ($p < 0.05$), while the CD4+/CD8+ coexpression was increased respect to baseline values. The discontinuation of CT was associated with a normalization of leucocytes,ANL and the CD16+, CD56+ subsets and circulating DCs values. During follow-up the CD8+ was increased with respect to the baseline and the CD4+ significantly decreased ($p = 0.01$). Finally the lymphocytes B subset was statistically decreased for at least one months ($p < 0.05$).On the basis of these results, the intensification of CT including A/T with Peg-FIL support does not have a negative impact on the immune system of CT-naïve BC pts.

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NATURAL KILLER (NK) AND DENDRITIC CELLS (DCS) PROFILE IN ADVANCED COLORECTAL CANCER PATIENTS TREATED WITH CHEMOTHERAPY + CETUXIMAB

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In order to observe the profile of the expression of circulating NK and DCs and to correlate it with time to progression, we studied patients with advanced colorectal cancer treated with cetuximab plus irinotecan after the failure of conventional chemotherapy. From December 2003 to September 2004, 11 patients were treated with weekly cetuximab, monoclonal antibody anti-EGFR (Merck), and biweekly irinotecan for progressing advanced colorectal cancer (MABEL protocol). All patients received the same dosage of irinotecan plus weekly cetuximab until disease progression (three monthly evaluation): 8 patients (males/females: 6/2; Karnofsky PS: >80%; median age: 61, range: 42–64 years) were evaluable. The immunophenotype has been determined on peripherical lymphocytes, and circulating DCs were sorted among mononuclear cells: in particular we observed the NK (CD56+) and total DCs expression before and after three or more months of treatment with cetuximab and irinotecan. Four patients had stable disease and four had progressive disease. The results obtained are represented in table 1:

	% Circulating NK/DCs median value (n.v.: 0–5 / 0,6–1,2%)		
Patients (n)	Time 0*	+ 3 m*	> 3 m*
Total (8)	15,2 / 0,6	12,5 / 0,3	15,6 / 0,6
Responders (4)	11,6 / 0,6	12,5 / 0,7	17,6 / 0,8
Non responders (4)	18,9/ 0,3	12,6/ 0,5	6,6/0,6

*time 0: before treatment; +3 m: after three months of treatment ; >3 m: after more than three months of treatment

°one valuable patient.

In this small study it seems that the increasing expression of circulating NK and DCs could be correlated with the response to cetuximab as stable disease after twelve or more weeks of treatment: prospective studies are necessary to confirm these data.

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CHROMATIN ASSEMBLY FACTOR-1 (CAF-1) AND DNA-PLOIDY IN PROGNOSTIC EVALUATION OF TONGUE SQUAMOUS CELL CEARCINOMA

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Squamous cell carcinoma (SCC) is the most frequent malignant tumor of the oral cavity. The tongue constitutes the most frequent localization of oral SCC, with an incidence of 27,6%. TSCC shows an aggressive biologic behaviour and a poor prognosis. The conventional clinical-pathological parameters predictive of prognosis, as tumour grade and stage, are still far to provide a conclusive outcome prevision for single cases of tumors. Recently, a great deal of interest has been delivered to the inter-relationship between chromatin organization, DNA damage processing and the "cell cycle checkpoint machinery." The resetting of the pre-existing chromatin structure during DNA synthesis, DNA replication, and/or DNA repair is at least in part related to the Chromatin Assembly Factor 1 (CAF-1), a molecular complex formed by three subunits (p48; p60; p150), which requires the interaction with the proliferating cell nuclear antigen (PCNA). Forty-six formalin-fixed, paraffin-embedded surgical specimens of primary TSCC, were retrieved from the files of the Department of Biomorphological and Functional Sciences, Section of Pathology, University Federico II of Naples and from the Polytechnic University of Marche (Ancona), from March 1990 to August 2002. 5 mm serial sections from routinely formalin fixed paraffin embedded blocks were cut for each case. CAF-1 expression was evaluated by immunohistochemistry with anti-p60 and anti- p150 (Ab-Cam U.S.A). A section from each case was stained with Feulgen's technique, to evaluate the DNA ploidy. p60 expression was compared with immunostaining for PCNA (PC10, DBA-Italy), and with the histopathological and follow-up data (49,9 months, mean) and resulted significantly higher in undifferentiated tumours and in advanced clinical stage. P60 was able to identify relapsing and/or metastasizing tumors. Moreover, a clear downtrend or absence of expression of p150 characterized this subclass of tumors with unfavourable behaviour, and the DNA index was able to discriminate the cases with nodal metastasis. The combined immunohistochemical evaluation of the expression of the CAF-1 p60 and p150 may contribute to the prognostic evaluation of TSCC. Tumors with low/absent expression of p150 and over expression of p60 may constitute a group of highly aggressive lesions, particularly when associated with high values of DNA index.

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INDUCTION OF RESISTANCE TO APLIDIN® IN A HUMAN OVARIAN CANCER CELL LINE RELATED TO MDR EXPRESSION

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Aplidin® resistant IGROV-1/APL cells were derived from the human ovarian cancer IGROV-1 cell line by exposing the cells to increasing concentration of Aplidin® for 8 months, starting from a concentration of 10 nM to a final concentra-

tion of 4 μM. IGROV-1/APL cell line possesses five fold relative resistance to Aplidin®. IGROV-1/APL resistant cell line shows the typical MDR phenotype: (i) increased expression of membrane-associated P-glycoprotein, (ii) cross-resistance to drugs like etoposide, doxorubicin, vinblastine, vincristine, taxol, colchicin and the novel anticancer drug Yondelis™ (ET-743). The Pgp inhibitor cyclosporin-A restored the sensitivity of IGROV1/APL cells to Aplidin® by increasing the drug intracellular concentration. The resistance to Aplidin® was not due to the other proteins, such as LPR-1 and MRP-1, being expressed at the same level in resistant and parental cell line. The finding that cells over-expressing Pgp are resistant to Aplidin® was confirmed in CEM/VLB 100 cells, that was found to be 5-fold resistant to Aplidin® compared to the CEM parental cell line.

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IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF ANTIGENIC MIMOTOPES OF SURFACE IMMUNOGLOBULIN FROM TRANSFORMED B CELLS

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The idiotypic determinants of surface Ig on B cells have unique aminoacid sequence and function as highly specific tumor antigens. These surface Igs are functional associated with transmembrane Igα-β transducing proteins in the B cell receptor (BCR) complex. In transformed B cells, such as B-cell lymphoma and Multiple Myeloma, antigenic epitopes induce a BCR crosslinking followed by cell apoptosis and may be innovative tools for the detection and treatment of B-cell malignancies. To this end, we screened random peptide libraries (RPLs) displayed on phages with purified Igs from a human myeloma cell line (IM9) and a murine B cell lymphoma cell line (A20) and identified pools of specific peptides. The binding of selected epitopes to the cognate Igs was evaluated by ELISA. The peptides binding specificity to target tumor cells was evaluated by flow cytometry by using biotinylated peptides. Biotinylated pIM9 and pA20 peptides, specifically, have recognized the cognate cell line, while control random peptides were negative. Also, the sensitivity of peptide bindings was evaluated by flow cytometry; the selected pIM9 and pA20 peptides recognized specifically as few as 0.5% tumor cells in a mixed cell population. Confocal microscopy revealed a peptide colocalization with the BCR complex and showed that peptides were internalized. Specific peptide binding to cells triggered apoptosis of A20 (55%) and IM9 (30%) cells as demonstrated by Annexin V-FITC/PI staining and by TUNEL assay and was associated with activation of Caspases 9, 3 and 7 with no involvement of Caspase 8.

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ANALYSIS OF MELAN-A/MART-1 ANTIGEN EXPRESSION DURING DIFFERENTIATION INDUCED BY CYANIDIN-3-O-β-GLUCOPYRANOSIDE IN HUMAN MELANOMA CELLS

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We have previously demonstrated that cyanidin-3-O-βglucopyranoside (C-3-G), a flavonoid belonging to the anthocyanin class, which is widely spread throughout the plant kingdom and commonly present in the human diet, is able to induce melanoma cell differentiation (1), characterized by dendritic outgrowth and increased ability to synthesize melanin pigment. In this study we investigated weather differentiated phenotype, induced by C-3-G treatment in melanoma cells, is correlated with the modulation of the melanocytic differentiation marker Melan-A/MART1. This antigen is expressed in melanocytes and melanoma cells and is recognized by cytotoxic T-lymphocytes, reason why it is of great interest for clinicians as a potential immunotherapeutic target and for pathologists as a possible diagnostic marker. The expression of the melanocytic differentiation marker Melan-A/MART1 antigen in TVM-A12 human melanoma cell line, obtained from a metastatic lesion, was investigated by: a) cytofluorimetric analysis, b) confocal laser scanning microscopy (CLSM) and c) Western Blot analysis, using the Melan-A (A103) mouse monoclonal antibody. Our results demonstrate that the acquisition of differentiated phenotype induced by C-3-G in human melanoma cells is correlated with an increased expression of Melan-A/MART1 antigen. C-3-G represents an attractive candidate in the development of novel strategies for both treatment and immunotherapy of melanoma through consumption of C-3-G in an appropriate cancer prevention diet or diet supplement.

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