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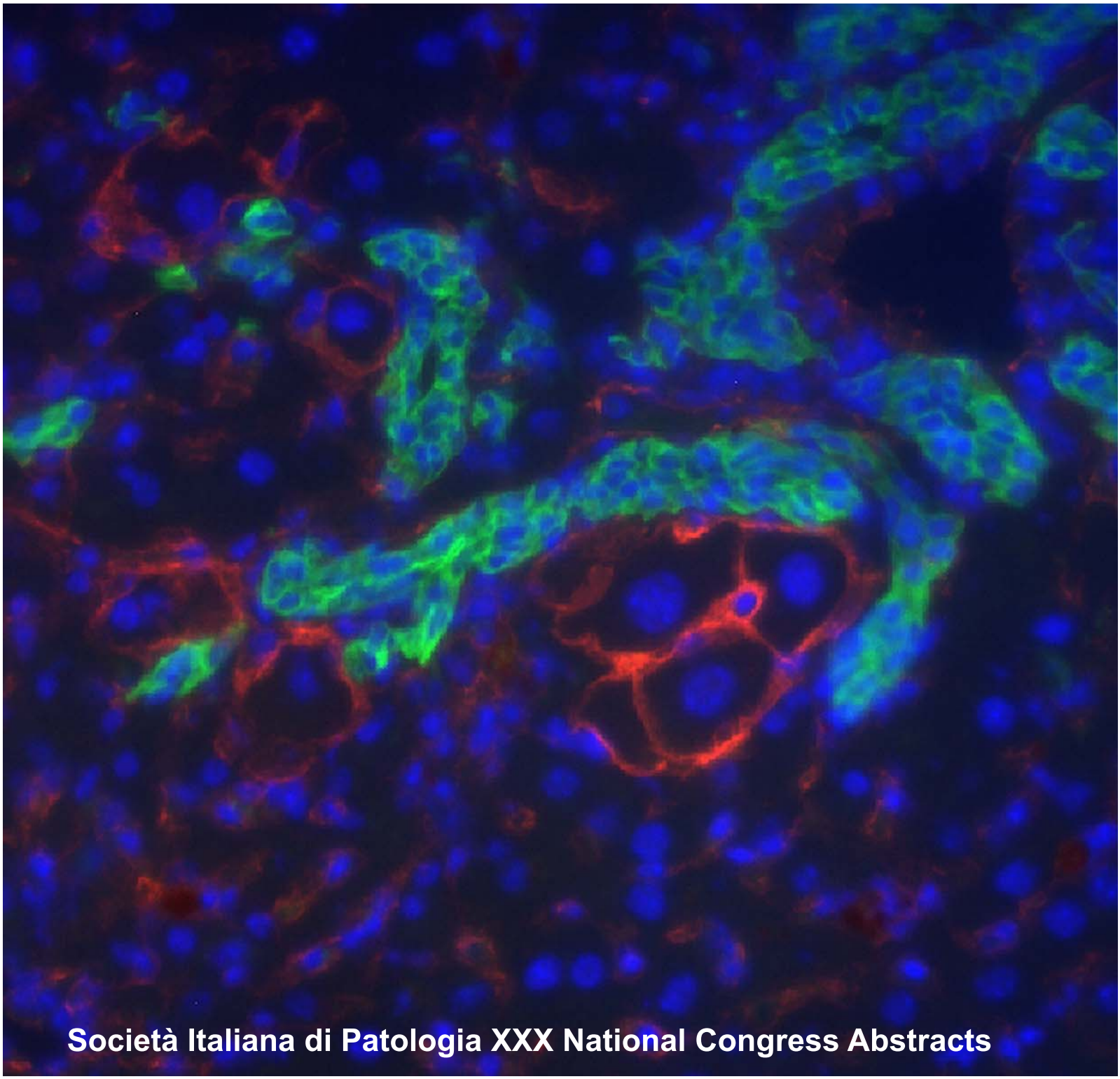
The American Journal of PATHOLOGY

Cellular and Molecular Biology of Disease

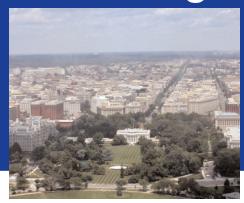
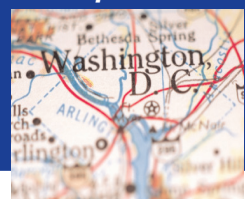


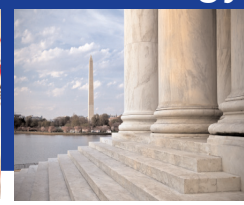
Società Italiana di Patologia XXX National Congress Abstracts

October 2010 Volume 177, Supplement

American Society for Investigative Pathology

2011 Annual Meeting at Experimental Biology



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April 9-13, 2011
Washington, DC (USA)

Abstract Submission Deadline:
November 8, 2010

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- American College of Veterinary Pathologists (ACVP)
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- International Society for Analytical and Molecular Morphology (ISAMM)
- International Society for Biological and Environmental Repositories (ISBER)
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Online Supplement to

The American Journal of Pathology

Official Journal of the American Society for Investigative Pathology

October 2010 ● Volume 177, Supplement

On the Cover: Administration of hepatotoxic diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) for 30 days to β-catenin conditional null mice results in appearance of a few β-catenin-positive hepatocytes (β-catenin staining; red) in the periportal region of the liver whereas a traditional stem cell source of hepatocytes resulting from atypical ductular proliferation (A6 staining; green) remains β-catenin negative. (See abstract SCR 05 on page S31.)

Meeting Abstracts

Società Italiana di Patologia XXX National Congress Abstracts

The XXX National Congress of Società Italiana di Patologia (Italian Society of Pathology),
in collaboration with the American Society for Investigative Pathology,
was held at the University of Salerno, Salerno, Italy, October 14-17, 2010.

To cite the Abstracts published here, please use the following format:

Barrera G, Cerbone A, Toaldo C, Pizzimenti S, Pettazzoni P, Dianzani C, Minelli R, Roma G, Dianzani MU, Ferretti C: AS601245, an Anti-Inflammatory JNK Inhibitor, Strengthens the Effects of Clofibrate and Affects Gene Expression Profile, in CaCo-2 Colon Cancer Cells. XXX National Congress of Società Italiana di Patologia, 2010 October 14-17, Salerno, Italy. Am J Pathol 2010, 177(Suppl):S1 Abstract ACT 01

These abstracts were reviewed by the Società Italiana di Patologia (Italian Society of Pathology) and the American Society for Investigative Pathology.
The American Journal of Pathology was not involved in the peer review process.

Società Italiana di Patologia

XXX National Congress Abstracts

October 14-17, 2010
University of Salerno, Salerno, Italy

ADVANCES IN MOLECULAR CANCER THERAPIES

ACT 01. AS601245, an Anti-Inflammatory JNK Inhibitor, Strengthens the Effects of Clofibrate and Affects Gene Expression Profile, in CaCo-2 Colon Cancer Cells.

G. Barrera[1], A. Cerbone[2], C. Toaldo[1], S. Pizzimenti[1], P. Pettazzoni[1], C. Dianzani[1], R. Minelli[1], G. Roma[2], M. U. Dianzani[1], C. Ferretti[1]
[1]University of Torino, Turin, Italy; [2]MerckSerono Ivrea – RBM S.p.A. - Istituto di Ricerche Biomediche, Turin, Italy

Background: Peroxisome proliferetor activated receptors (PPARs) are nuclear receptors activated by ligands. PPARα can be activated by the hypolipidemic drugs (i.e. clofibrate). PPARα transcriptional activity can be negatively regulated by JNK-mediated phosphorylation. We postulated that the use of a JNK inhibitor could increase the effectiveness of clofibrate.
Methods: We analysed the effects of 0.1 μM AS601245 (a selective JNK inhibitor) and 5 μM clofibrate alone or in association, on apoptosis (by analyzing caspase 3/7 activity) and differentiation (by analysing Domes formation) in CaCo-2 human colon cancer cells. Gene expression profile was evaluated by using Affimetryx analysis. The data are confirmed by real time PCR. The PPARα binding activity to DNA was performed by using Trans-AM ELISA-based kit.
Results: Apoptosis was only induced by the combined treatment with the two substances. Differentiation increased after treatment with AS601245 and, to a higher degree, after combined treatment. Some genes, involved in these phenomena, were highly modulated by the combined treatment, and 28 genes, containing peroxisome proliferators responsive elements (PPRE), were up-regulated. The analysis of the PPARα binding to PPRE demonstrated that it was strongly increased in cells treated with both compounds.
Conclusions: Our data demonstrated the effectiveness of combined treatments in inducing apoptosis and differentiation and in affecting gene expression in colon cancer cells.

ACT 02. Selective Inhibition of Carbonic Anhydrase IX Decreases Cell Proliferation and Induces Ceramide-Mediated Apoptosis in Human Cancer Cells.

F. Cianchi[1], M. C. Vinci[1], C. T. Supuran[1], B. Peruzzi[2], P. De Giuli[3], G. Fasolis[3], G. Perigli[1], S. Pastorekova[4], L. Papucci[1], N. Schiavone[1], A. Pini[1], E. Masini[1], L. Puccetti[1]
[1]University of Florence, Florence, Italy; [2]Istituto Toscano tumori, Florence, Italy; [3]Ospedale San Lazzaro, Alba, Italy; [4]Slovak Academy of Science, Bratislava, Italy

Background: Recently, carbonic anhydrase (CA) inhibitors have been proposed as a potential new class of antitumor agents. The aim of this study was to evaluate the antitumor activity of three CA inhibitors, namely, acetazolamide (AZ) and two newly synthesized aromatic sulfonamides with high affinity for CA IX, TR1 and GA15, against human tumor cells.
Methods: The effects of AZ, TR1 and GA15 on cell proliferation and apoptosis were evaluated in the CA IX-positive HeLa and 786-O cells and in the CA IX-negative 786-O/VHL cells. We also investigated whether the potential antitumor activity of these molecules might be mediated by an increase in ceramide production.
Results: AZ, TR1 and GA15 could significantly reduce cell proliferation and induce apoptosis in the HeLa and 786-O cells. Moreover, all three inhibitors could decrease intracellular pH (pHi) and increase ceramide production in the same cells. Treatment with the ceramide synthase inhibitor fumonisin B1 prevented the apoptotic effects of the three CA inhibitors. In all experiments, the effects of aromatic sulfonamides were more pronounced than those of AZ. The three inhibitors did not show any antitumor activity in the CA IX-negative 786-O/VHL cells and failed to lower pHi and increase intracellular ceramide levels in the same cells.
Conclusions: In conclusion, CA inhibition can decrease cell proliferation and induce apoptosis in human tumor cells. The ability of CA inhibitors to decrease pHi might trigger cell apoptosis through mediation of ceramide synthesis. Activation of this apoptotic cascade is most likely mediated by inhibition of the CA IX isoform.

ACT 03. Effects of N6-Isopentenyladenosine and Analogues on Bladder Cancer Cells

R. Ottria[1], S. Casati[1], E. Baldoli[1], J. Maier[1], P. Ciuffreda[1]
[1]Università di Milano, Milano, Italy

Background: Isopentenyladenosine (iPA), the only known cytokinin in animal cells, is a modified nucleoside with a pentaatomic isopentenyl chain that binds the nitrogen at the position 6 of the purinic base. iPA exerts a potent antiproliferative activity on tumour epithelial cells. To obtain cytostatic iPA derivatives potentially useful as chemotherapeutic agents, we synthesized a series of adenosine analogues differently substituted in N6-position and evaluated them in vitro.
Methods: We synthesized adenosine analogues modified in N6-position with a saturated linear chain, one or more hydroxyl or amino groups on the N6-substituent, an unsaturated chain, a cyclic substituent and an aromatic ring. We evaluated their activity on T24 and J82 bladder carcinoma cell growth, motility and invasivity in matrigel assays.
Results: Out of 20 analogues synthesized, only N6-benzyladenosine inhibited T24 cell growth as much as iPA, while N6-allyladenosine, N6-cyclobutylmethyladenosine and N6-(3-methylbutyl)-adenosine were less bioactive. Neither iPA or its analogues had any effect on cell migration, synthesis of matrix metalloproteases and invasiveness.
Conclusions: These results indicate that modifications of N6-position impact on the cytostatic activity of iPA. On the contrary, we have shown that the modifications of the sugar moiety or the purine base of iPA generate inactive molecules. We also show that iPA and its analogues have no effect on the migration and the invasivity of bladder cancer cells.

ACT 04. Novel Human Anti-ErbB2 Immunoagents

C. De Lorenzo[1], C. Fedele[2], E. Malara[1], F. Troise[1], G. Riccio[1], P. Laccetti[1], G. D'Alessio[1]
[1]Università di Napoli Federico II', Napoli, Italy; [2]Università di Napoli, Napoli, Italy

Background: Overexpression of ErbB2 receptor is a sign of malignancy and poor prognosis of breast cancer. Herceptin, a humanized anti-ErbB2 antibody, has proved to be effective in the therapy of breast carcinoma, but it can engender cardiotoxicity and many breast cancer patients are resistant to Herceptin treatment.Two novel human antitumor immunoconjugates were engineered in our laboratory by fusion of a human anti-ErbB2 scFv, termed Erbicin, with either a human RNase or the Fc region of a human IgG1. Both Erbicin-Derived Immunoagents immunoagents (EDIA) are selectively cytotoxic for ErbB2-positive cancer cells in vitro and vivo.
Methods: The finding that EDIA recognize an epitope different from that of Herceptin led to ascertain whether they might not present the most negative properties of Herceptin: cardiotoxicity and inability to act on resistant tumors.
Results: EDIA did not show cardiotoxic effects both *in vitro* on rat and human cardiomyocytes and *in vivo* on a mouse model, whereas Herceptin was strongly toxic. This difference was found to be due to their different mechanism of action: Herceptin, at difference with Erb-hcAb, induces apoptosis in cardiac cells.More interestingly, EDIA were active on some Herceptin-resistant cancer cells both *in vitro* and *in vivo*. The sensitivity of these cells to treatment with EDIA is likely due to their different epitope, since EDIA, at difference with Herceptin, was able to inhibit the signalling pathway downstream ErbB2.
Conclusions: EDIA could fulfil the therapeutic need of cancer patients ineligible for Herceptin treatment due to cardiac dysfunction and to primary or acquired Herceptin-resistance.

ACT 05. BCR-ABL Tyrosine Kinase Activity Modulates the Phosphorylation, Localization and Function of Interferon Regulatory Factor 5 (IRF-5) in Chronic Myeloid Leukemia Cells

L. Manzella[1], M. Massimino[1], A. Fidilio[1], M. L. Consoli[1], P. Buffa[1], M. Fallica[1], P. Vigneri[1], A. Messina[1]
[1]University of Catania, Catania, Italy

Background: IRF-5, is a trancription factor which has tumor suppressor properties. In fact, it regulates the expression of several genes including Bak, Bax, p21, caspase-8. The aim of this research was to study the relationship between IRF-5 and the BCR-ABL oncoprotein of Chronic Myeloid Leukemia (CML).

Methods: We assessed IRF-5 expression by immunoblot on both primary cells and three immortalized cell lines: K562, KCL22, KYO-1. The association between IRF-5 and BCR-ABL was investigated by immunoprecipitation assays while IRF-5 intracellular localization was studied through fractionation experiments. Growth in methylcellulose was used to determine the transforming activity of CML cells expressing IRF-5 constructs.
Results: We found that: IRF-5 is expressed in both primary and immortalized CML cells, is associated with BCR-ABL and is a target of the oncoprotein kinase activity. In CML cells, IRF-5 was mostly confined to the cytoplasm. However, treatment with Imatinib Mesylate (IM) or with alpha-Interferon relocalized IRF-5 to the nucleus suggesting that BCR-ABL modulated its nuclear-cytoplasmic shuttling. Mutagenesis of IRF-5 tyrosine 104 to phenylalanine generated a mutant that displayed reduced levels of tyrosine phosphorylation. In addition, IRF-5 Y104F mainly localized to the cell nucleus. Finally, over-expression of IRF-5 Y104F reduced proliferation and foci formation of both CML cell lines and CD34+ cells isolated from CML patients.
Conclusions: Our findings demonstrate that BCR-ABL associates with IRF-5 and causes its phosphorylation on tyrosine 104, thereby preventing IRF-5 nuclear localization and transcriptional activity. Strategies aimed at disrupting the interaction between BCR-ABL and IRF-5 may represent a novel approach to reduce the proliferation of CML.

ACT 06. Bergapten Inducing Proteasome-Dependent Degradation of Estrogen Receptor α May Overcome Breast Cancer Tamoxifen-Resistance

M. L. Panno[1], F. Giordano[1], V. Bartella[1], S. A. W. Fuqua[2], F. De Amicis[1], M. Maggiolini[2], S. Ando[1]
[1]University of Calabria, Arcavacata di Rende, Italy; [2]Breast Center, Baylor College of Medicine, Houston, TX, USA

Background: Bergapten, a psoralen bioactive compound, induces antiproliferative and pro-apoptotic actions in breast and in other tumoral cells. However, it remains to be investigated if the above reported events may come from Bergapten interfering on E2/ERα stimulatory signalling. To ascertain the latter concern we evaluated ERα cell compartmentalization upon Bergapten exposure in two estrogen receptor α-positive breast cancer cells: MCF-7 and ZR-75.
Methods: MTT assay was used to evaluate the effect of psoralen on cell growth in breast cancer cells, while protein expressions were detected by Western Blot. Cytosolic and nuclear fractions were also prepared by extraction with hypotonic and hypertonic lysis buffers. Reverse Transcriptase–Polymerase Chain Reaction Assay was performed for ERα mRNA expression.
Results: It is worth to note that psoralen treatment, in the presence of unchanged ERα mRNA, produces a decrease of ERα content, mainly localized in the cytosolic compartment, concomitantly with a strong lowering of cyclin D1. These effects were reversed by the proteasoma inhibitor MG132, sustaining how the decrease of ERα was due to an enhanced protein ubiquitination. The described effects induced by Bergapten were reproduced in MDA-MB-231 cells ectopically expressing ERα. However, the most striking finding was that in MCF-7 Tamoxifen resistant cell line psoralen “per se” was able to reverse the stimulatory action of anti-estrogen.
Conclusions: We conclude that Bergapten may be considered as a potential anti-tumoral agent to be exploited in the novel therapeutical strategies for breast cancer developing hormone-resistance.

ACT 07. PPAR-γ Ligand Rosiglitazone Counteracts the Stimulatory Effect of Leptin on Breast Cancer Growth

M. Pellegrino[1], S. Catalano[1], D. Bonofiglio[1], H. Qi[1], P. Rizza[1], D. Vizza[1], G. Bossi[2], L. Mauro[1]
[1]University of Calabria, Arcavacata di Rende, Italy; [2]Regina Elena Cancer Institute, Roma, Italy

Background: Obesity is a major risk factor for the development and progression of breast cancer. Leptin, a cytokine mainly produced by adipocytes, plays a crucial role in mammary carcinogenesis and is elevated in hyperinsulinemia and insulin-resistance. The antidiabetic thiazolidinediones inhibit leptin gene expression through ligand-activation of the Peroxisome Proliferator-Activated Receptor-gamma (PPAR- γ) and exert antiproliferative and apoptotic effects on breast carcinoma.
Methods: In vivo experiments were performed in MCF-7 cell tumor xenografts implanted in female nude mice. The expression of leptin, ObR, aromatase and different classic estrogen genes was studied by RT-PCR. The activation of leptin signaling pathway components was evaluated with western blotting analysis. Leptin and BRL effects on leptin promoter transcription were studied by luciferase reporter assays. Functional studies on leptin promoter were done by EMSA, ChIP and Re-ChIP assays.
Results: We show that PPAR- γ ligand rosiglitazone (BRL) is able to reverse leptin-stimulated breast tumor growth *in vivo* and *in vitro* models. BRL down-regulates the increase of leptin gene expression, its receptors and inhibits MAPK/STAT3/Akt phosphorylation induced by leptin. PPAR- γ -mediated repression of leptin gene involves the recruitment of NCoR/SMRT corepressors as components of a multiprotein complex including Glucocorticoid Receptor and PPAR- γ. In addition, we demonstrated that PPAR-γ activation counteracts leptin stimulatory action on estrogen signaling in breast cancer.
Conclusions: These findings suggest that PPAR- γ ligands may have potential therapeutic benefit in the treatment of breast cancer particularly in obese women.

ACT 08. New Strategies to Overcome Resistance to EGFR Tyrosine Kinase Inhibitors in Non Small Cell Lung Cancer Cell Lines

R. Alfieri[1], A. Cavazzoni[1], M. Galetti[1], R. Andreoli[1], C. Carmi[1], M. Mor[1], P. G. Petronini[1]
[1]University of Parma, Parma, Italy

Background: Epidermal Growth Factor Receptor (EGFR) is an established new target for the treatment of non-small cell lung cancer (NSCLC). Small molecule inhibitors, such as erlotinib and gefitinib, have proven to be a useful addition to standard therapy in advanced NSCLC. However, tumor cells often acquire resistance to these EGFR inhibitors. In this study we have investigated new therapeutic approaches to circumvent acquired gefitinib resistance.
Methods: Experiments were performed on a panel of NSCLC cell lines. Cell proliferation, cell viability, apoptosis and signal transduction pathways were evaluated by standard methodologies. Gefitinib uptake, efflux and metabolism were evaluated by LC-MS/MS.
Results: We assessed the involvement of constitutive activation of the PI3K/AKT signaling pathway in resistance to gefitinib and we demonstrated that a combined treatment with gefitinib and Everolimus (mTOR inhibitor) induced a significant decrease in the activation of MAPK and mTOR pathways and resulted in a growth-inhibitory effect in resistant cell lines. The design and synthesis of a new generation of EGFR reversibile and irreversible inhibitors with an improved *in vitro* potency and selectivity were other rational approaches we used to treat acquired resistance to gefitinib. Finally we have characterized the gefitinib transport system and we have evaluated whether the modulation of intracellular concentration of gefitinib may overcome gefitinib resistance.
Conclusions: Irreversible EGFR TKI characterized by novel reactive group have been validated.This study should have an impact on clinical practice by leading to a more focused and rationale use of molecular targeted therapies with EGFR-TKI inhibitors.

ACT 09. Lung Cancer: Estrogen Receptors and 4-Hydroxytamoxifen Activity

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[1]University of Bologna, Bologna, Italy; [2]Istituto Ortopedico Rizzoli, Bologna, Italy

Background: Studies on sex differences in lung cancer risk and disease presentation suggest that estrogen-signalling pathways may play a key role in the genesis and in controlling the growth of lung cancer. Cellular response to estrogen is mediated by ERα and ERβ. Recently, the GPR30 receptor has been implicated in rapid and specific estrogens binding in mediating the action of several estrogenic compounds. In this work, we analized the ER pathway including GPR30, in human metastatic lung cancer with respect to the activity of a selective estrogen receptor modulator, 4-OHT
Methods: Human cell lines RAL and SCLC-R1 were obtained from metastatic lesions of lung adenocarcinoma and of small cell lung carcinoma respectively and grown in H/H medium+ 10% FBS. Growth inhibition by TBDE and protein expression by WB were tested.
Results: In each cell line, results indicate (1) the expression of GPR30 and ERβ and not ERα; (2) a significant increase in cellular growth of the cells exposed to estradiol(E2);(3) that 4-OHT antiestrogen causes a significant dose- and time-dependent growth inhibition at therapeutic doses.
Conclusions: The present data indicate for the first time that lung RAL and SCLC-R1 cell lines express ERβ and GPR30 and not ERα. This is in line with previous data indicating opposite roles for ERα and GPR30 in the control of cell proliferation in most cancers. In addition, results show that the growth of these cell lines is sensitive to estrogen stimulation. The antiestrogen 4-OHT, effective here in inhibiting cell growth, may warrant attention for future utilization in lung cancer therapy.

ACT 10. Expression and Role in the Proliferation of the Colony-Stimulating Factor-1 Receptor in Breast Cancer Cells

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[1]Università di Firenze, Firenze, Italy

Background: Breast cancer is the most prevalent cancer in non-smoking women and the second leading cause of cancer-related deaths in western countries. The Colony-Stimulating Factor 1 (CSF1) and its receptor CSF1R physiologically regulate the monocyte/macrophage system, trophoblast implantation and breast development. An abnormal expression of CSF1R, associated or not with that of CSF1, has been also documented in several human epithelial tumors, including breast carcinomas. Despite the fact that the expression of both CSF1 and CSF1R strongly correlates with poor prognosis of breast cancer, scanty data have been obtained on CSF1R signaling in neoplastic cells.
Methods: 17 breast cancer cell lines of different molecular classes (i.e. presence/absence of estrogen or progesterone receptors or HER2 over-expression) were used.
Results: All 17 cell lines tested but one expressed cell surface CSF1R protein or CSF1R mRNA although to different extents. Moreover, in silico analysis from microarray data of 49 breast cancer cell lines indicated that CSF1R mRNA was consistently expressed, irrespectively of molecular class. siRNA silencing of CSF1R or blocking CSF1 with an

anti-serum in SKBR3 cells markedly decreased, while exposure of MDAMB468 cells to CSF1 increased, CSF1-dependent cell proliferation. We also found that ERK1/2, c-Jun, Cyclin D1 and c-Myc are CSF1/CSF1R targets in breast cancer cells. Imatinib decreased cell prolifaration in the presence of CSF1 of a number of cell lines.
Conclusions: Taken together, the results reported here indicate that CSF1 and CSF1R are determinant for the proliferation of breast cancer cells and point to CSF1R as a possible new target for breast cancer therapy.

ACT 11. Estrogen Receptor β Induces Apoptosis in TCAM2 Human Seminoma Cell Line.

C. Guido[1], S. Panza[1], P. Avena[1], I. Casaburi[1], F. Giordano[1], M. Bifulco[2], S. Andò[1], S. Aquila[1] .
[1]University of Calabria, Arcavacata di Rende, Italy; [2]University of Salerno, Salerno, Italy

Background: Testicular germ cell tumors of adults and adolescents (TGCTs) are the most common tumor in male. TGCTs can be classified into two main histological subtypes, seminoma (SE) and nonseminoma (NS). Here the focus is on SE, by using the TCam-2 cell lines, containing typical features of human seminoma and originated from a primary testicular seminoma of a 35-year-old patient. Testicular cancer research continues to modify current therapies aimed to induce cancer cell apoptosis. An important mammalian cell survival signalling is mediated by phosphoinositide 3-OH kinase (PI3K) and its downstream targets.
Methods: To gain more insight in the biology of testicular tumours by western blotting and transient transfection assays, we studied the effect of increasing E2 concentrations on the PI3K pathway.
Results: Our data evidenced that E2 was able to increase PTEN expression as well as caspase-9 and PARP-1 cleavages. Concomitantly the expression of Akt, FHKR, BAD decreased suggesting an apoptotic role of the hormone through the ERβ, since our cells do not express the ERα. Silencing the ERβ the effect was abrogated, while overexpressing ERα an increased expression of Akt was obtained.
Conclusions: Although further work is required to clarify the mechanism/s through which E2 induces apoptosis in human seminoma, our study suggest a tumor suppressor role for the ERβ. Concluding, these preliminary data support estrogen-dependency of human testicular seminoma and candidate the ERβ-ligands for a therapeutic tool in the treatment of this pathological condition.

ACT 12. Analysis of Tyrosine Kinase Receptor Expression in Endometrial Stromal Sarcoma

M. R. Muroni[1], P. Cossu Rocca[1], M. Contini[1], M. G. Uras[1], A. Mura[1], L. Murgia[1], S. Ena[1], C. Carru[1], L. Bosincu[1], M. R. De Miglio[1]
[1]University of Sassari, Sassari, Italy

Background: Endometrial stromal sarcomas (ESS) are rare neoplasms, currently classified into low grade (LG) ESS, and undifferentiated endometrial sarcomas (UES). Surgery remains the treatment of choice for ESS, whereas effective adjuvant therapies have not yet been established. Thus, alternative approaches such as molecularly targeted therapies need to be investigated. The aim of our study was to analyze immunohistochemical expression of tyrosine kinase receptors in a ESS series, to evaluate their potential role as molecular targets.
Methods: Immunohistochemistry was performed in 10 ESS, i.e. 7 LG-ESS and 3 UES. Specific antibodies against ABL, CD117, EGFR, PDGFR-α have been used. Staining intensity and % of positive cells were scored for each case.
Results: ABL expression was detected in 5 out of 7 LG-ESS and 2 out of 3 UES, with % of positive cells ranging from 10 to 50%, and staining intensity ranging from 1+ to 2+. EGFR expression was observed in 6 out of 7 LG-ESS and all the 3 UES, with % of positive cells ranging from 50 to 80%, and staining intensity ranging from 1+ to 3+. PDGFR- α expression was detected in 6 out of 7 LG-ESS and all the 3 UES, with % of positive cells ranging from 30 to 70%, and staining intensity ranging from 1+ to 2+. CD117 expression was consistently negative in all the cases.
Conclusions: Our study confirms that tyrosine kinase receptor expression is frequent in ESS. Further studies are needed to identify specific genetic abnormalities, potentially useful to select patients who might benefit from targeted therapies.

ACT 13. Computational and Experimental Characterization of Critical Amino Acid Residues in the BCR-ABL Kinase Domain Explaining Imatinib Resistance in Patients with Chronic Myeloid Leukemia

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Background: Inhibition of BCR-ABL catalytic activity by Imatinib Mesylate (IM) has dramatically improved the natural history of Chronic Myeloid Leukemia (CML) ushering the era of molecular targeted therapy. Despite the unprecedented results achieved by IM, recent findings suggest that 20-30% of CML patients will fail the drug and, after 10 years, up to 50% will require alternative treatment strategies. Thus, the need for a structural characterization of the interactions between the BCR-ABL kinase domain and IM, that will define the mechanisms allowing BCR-ABL mutants to escape the activity of the compound.
Methods: We have computationally characterized six amino acids that appear critical for IM binding. We next generated tagged-BCR-ABL constructs displaying conservative or non-conservative mutations in each of these amino acidic residues. Every BCR-ABL mutant has been lentivirally transduced in BaF3 cells and evaluated for expression, catalytic activity, trasforming potential and response to IM or to second-generation tyrosine kinase inhibitors (TKIs).
Results: Conservative mutations in IM-binding residues (IBRs) preserved BCR-ABL kinase activity and transforming potential, but also maintained TKI binding. Non-conservative substitutions drastically altered the three-dimensional conformation of the BCR-ABL catalytic domain, compromising the oncoproteins kinase activity. In either case, these mutants failed to confer a selective advantage to leukemic cells.
Conclusions: Our findings have identified the minimal binding region necessary for the interaction between BCR-ABL and IM and should aid in the design of novel kinase inhibitors for the treatment of CML patients failing TKIs because of point mutations in the BCR-ABL catalytic domain.

ACT 14. Caveolin-1 Silencing Blocks Metastatic Lung Cancer Proliferation *In Vitro*

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Background: Caveolin-1 (cav-1) is an essential structural constituent of caveolae implicated in mitogenic signalling, oncogenesis, angiogenesis, neurodegenerative diseases and senescence. It is highly expressed in some tumours *in vivo*, including lung adenocarcinoma, and this is associated with increased tumor aggressiveness, metastatic potential and suppression of apoptosis.
Methods: Human cell lines RAL and SCLC-R1 were obtained by us from metastatic lesions of lung adenocarcinoma and of small cell lung carcinoma respectively and grown in H/H medium+ 10% FBS. Inhibition of Cav-1 expression was performed by the use of small interfering RNA (siRNA). Growth inhibition by TBDE and protein expression by WB were tested.
Results: Results indicate that (1) lung RAL and SCLC-R1 metastatic cells express high levels of cav-1 protein; (2) a SiRNA-mediated down-regulation of caveolin-1 expression is evident in SCLC-R1 (100%) and RAL (80%) cells; (3) cav-1 knockdown causes arrest of cell growth in both cell lines, maintained up to 72 h after transfection; (4) Cav-1 inhibition affects the expression of cell cycle regulatory proteins and thereby cell cycle progression.
Conclusions: A growing body of evidence links elevated cav-1 expression to an aggressive malignant and metastatic phenotype in several tumors. This has been recently reported in lung adenocarcinoma. The present data indicate for the first time that lung RAL and SCLC-R1 cell lines express high levels of cav-1 and demonstrate that cav-1 knock-down arrests metastatic growth either in small cell lung carcinoma or in adenocarcinoma *in vitro* by a novel molecular pathway.

ACT 15. Rosiglitazone, Alone or in Combination with AS601245, an Anti-Inflammatory JNK Inhibitor, Affects Apoptosis, Differentiation and Cell Adhesion in CaCo-2 Human Colon Cancer Cells Through Modulation of Specific Gene Expressions.

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Background: Rosiglitazone, an antidiabietic drug, is a synthetic ligand for PPARγ. In colon cancer cells, activation of PPARγ leads to reduction of proliferation and induction of apoptosis. PPARγ transcriptional activity can be negatively regulated by JNK-mediated phosphorylation. The use of an agent able to inhibit JNK activity, could increase the effectiveness of PPARγ ligands
Methods: We analysed the effects of 50 μM rosiglitazone and 0.1 μM AS601245 (a JNK inhibitor) alone or in association, on apoptosis differentiation and adhesion of CaCo-2 human colon cancer cells. Moreover, by using the Affimetryx analysis, we analysed gene expression in control and drug-treated cells.
Results: Apoptosis was induced by the combined treatment with the two substances, only. Differentiation was increased after treatment with rosiglitazone and, to a higher degree, after combined treatment. Gene expression analysis revealed some genes, highly modulated by the combined treatment, which could be involved in these phenomena. Rosiglitazone strongly reduced cell adhesion, AS601245 was less effective, and combined treatment resulted in the greatest reduction of the adhesion. Rosiglitazone down-regulated the expression of the three fibrinogen chains (α, β, γ) which were further down-regulated by the combined treatment. Moreover, rosiglitazone and combined treatment caused a decrease of fibrinogen release which paralleled with the decrease of

cell adhesion. The number of genes containing PPAR-binding sequences, activated by rosiglitazone, were increased after combined treatment.
Conclusions: Our data demonstrated the effectiveness of combined treatments with a PPARγ agonist and a JNK inhibitor, in inducing apoptosis and differentiation and in reducing cell adhesion in colon cancer cells.

ACT 16. CSF1 Determines CSF1R Cytoplasmic Nuclear Shuttling in Breast Cancer Cells

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Background: Breast cancer is the most prevalent cancer in non-smoking women and the second leading cause of cancer-related deaths in western countries. The Colony-Stimulating Factor 1 (CSF1) and its receptor CSF1R physiologically regulate the monocyte/macrophage system, trophoblast implantation and breast development. An abnormal expression of CSF1R, associated or not witht that of CSF1, has been also documented in several human epithelial tumors, including breast carcinomas. Despite the fact that the expression of both CSF1 and CSF1R strongly correlates with poor prognosis of breast cancer, scanty data have been obtained on CSF1R signaling in neoplastic cells.
Methods: Breast cancer cell lines or tissue samples of different molecular classes (i.e. presence/absence of estrogen or progesterone receptors or HER2 over-expression) were used to study the intracellular localization of CSF1/CSF1R by confocal microscopy, cell fractionation or immuno-histochemistry.
Results: We found that CSF1R is localized in the nucleus and nucleolus of breast cancer cells, both in cell lines and tissue samples and irrespectively of the molecular class of breast cancer, and that this localization depends on CSF-1. Moreover, we found by chromatin immunoprecipitation assay that CSF1R binds the promoters of cyclin D, c-myc and c-Jun, genes known to be regulated by CSF1/CSF1R and relevant for CSF1-induced cell proliferation.
Conclusions: The identification of a new mechanism of response to CSF1 in breast cancer cells points to the relevance of CSF1R in breast cancer development and treatment.

ACT 17. MYBL2 Expression is Under Genetic Control and is Involved in the Rapid Development of Hepatocellular Carcinoma.

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Background: We investigated the role of MYBL2 in acquisition of susceptibility to hepatocarcinogenesis and progression of preneoplastic and neoplastic liver, as well as the molecular mechanisms involved.
Methods: MYBL2 mRNA and protein were evaluated by qRT-PCR and Western blot. MYBL2 expression of hepatocellular carcinoma (HCC) cell lines was manipulated by MYBL2 cDNA or anti- MYBL2 siRNA transfection. Gene expression profile of MYBL2 transfected cells was analyzed by microarray.
Results: Low upregulation of MYBL2 and its target Clusterin, in low-grade dysplastic nodules (DN), progressively increased in fast growing high-grade DN and HCC of F344, susceptible to HCC, whereas lower increases occurred in slow growing lesions of resistant BN rats. Highest activation of MYBL2 protein, prevalently nuclear, occurred in F344 than BN lesions. Highest MYBL2 expression occurred in fast progressing DN and HCC of E2f1 transgenic mice, compared to low progressing lesions of Myc transgenics, and anti- MYBL2 siRNA had highest anti-proliferative and apoptogenic effects in cell lines from HCC of E2f1 transgenics. Highest expression of MYBL2 and MYBL2 target Cyclin B1 occurred in hHCC with poorer prognosis. MYBL2 transfected HepG2 and Huh7 cells exhibited increase in cell proliferation and G1-S and G2-M cell cycle phases. The opposite occurred in siRNA transfected cells. It was found that MYBL2 promotes survival of p53 mutant HCC cells when treated with doxorubicin as a DNA damaging agent.
Conclusions: MYBL2 expression and activation are under genetic control. MYBL2 upregulation induces fast growth and progression of premalignant and malignant liver. Expression and function inhibition of MYBL2 may be clinically relevant.

ACT 18. Interaction of Endocannabinoid System and Steroid Hormones in the Control of Colon Cancer Cell Growth

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Background: The cannabinoid CB1 and CB2 receptors are G-protein-coupled receptors able to control cell survival and death and to modulate signal transduction pathways involved in tumor progression, neoangiogenesis and metastases. Moreover, increasing evidence suggested a fine regulation of the cannabinoid receptor expression in cancer models.
Methods: In this study we focused our attention on the role of CB1 receptor, endocannabinoids and steroids in the mechanisms underlying inhibition of the colorectal cancer cell growth, *in vitro*.
Results: We demonstrated that 17β-estradiol induced a specific and strong up-regulation of CB1 receptor, in DLD1 and SW620 colon cancer cell lines. Physiological and pharmacological doses of 17 β -estradiol were both able to elicit the activation of CB1 promoting region, localized at the exon 1 of the CNR1 gene, by involvement of the genomic and non genomic pathways triggered by steroids, respectively. Moreover, treatment of DLD1 and SW620 with exogenous Met-F-AEA (10 microM), a stable AEA-analogous, or with URB597, a selective inhibitor of the FAAH, able to increase levels of endogenous AEA, induced up-regulation of CB1 expression by co-localization of PPARγ and RXRα at the promoting region. Finally, increased availability of AEA, of both exogenous and endogenous source, induced the expression of Estrogen Receptor-β in both cell lines.
Conclusions: Our results partially elucidated the role of endocannabinoid system in the molecular mechanisms enrolled by steroids in the inhibition of colon cancer cell growth and strongly suggested that targeting the cannabinoid system could represent a promising tool, at least to improve the efficacy of colorectal cancer treatment.

ACT 19. Study of the CB1 Receptor-Mediated Molecular Mechanisms, Regulated by Steroids and Endocannabinoids in Breast Cancer Cells

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Background: The Endocannabinoid System (ES) encompasses the complex signaling system of cannabinoid receptors, endogenous ligands and the enzymes responsible for their biosynthesis and inactivation. There is increasing evidence that endocannabinoids are able to inhibit cancer cell growth in culture as well as in animal models. However, endocannabinoids are now emerging as suppressors of angiogenesis and tumor spreading pointing to a potential role of the ES as a target for a therapeutic approach of such malignant diseases. It is a priority to identify new targets for drug development, either for those tumors that are insensitive to the most common treatments or to cooperate with other treatments to improve efficacy and avoid recurrence and resistance.
Methods: In this study we analysed the interaction between steroid hormones and ES in human breast cancer.
Results: AEA and Rimonabant inhibited the proliferation of MCF7 and MDA-MB231 and induced an increased expression of CB1 receptor. The anti-proliferative effect was preserved in the presence of both physiological and pharmacological doses of 17β-estradiol. Moreover, ChIP analysis of the CB1 promoting regions showed that, in these models, CNR1 behave like an estrogen inducible gene. Aimed to dissect the molecular mechanism responsible of the observed results, silencing experiments of both CB1 and alpha isophorm of oestrogen receptor have been also performed.
Conclusions: Although further work is required to clarify the mechanism through which 17β-estradiol induces CB1 receptor in human breast cancer cells our study strongly suggests a role for the ES and steroid modulated pathways in the control of breast cancer cell growth.

ACT 20. Overcoming Chemotherapy Resistance in Childhood Acute Lymphoblastic Leukemia by Targeting Ion Channels

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Background: Despite improvements in cure rates, chemotherapy resistance remains a major obstacle to successful treatment in a significant proportion of children with acute lymphoblastic leukemia (ALL), particularly in those with relapsed ALL. Bone marrow mesenchymal cells (MSC) can contribute to generate drug resistance in leukemic cells and several mechanisms have been proposed to explain this effect.
Methods: We studied the molecular mechanisms underlying the effects of bone marrow mesenchymal cells (MSCs) on supporting the growth and protecting from chemotherapy of acute lymphoblastic leukemia (ALL) cells. Coculture of leukemic cells with MSCs induced the expression of a signaling complex on the lymphoblast plasma membrane that consisted of hERG1 channels, the β1 integrin subunit and the chemokine receptor CXCR4.
Results: We found that this protein complex triggered the activation of both the ERK 1/2 and PI3K/Akt prosurvival signaling pathways. Indeed, the leukemic cells became markedly resistant to chemotherapy-induced apoptosis when cultured on MSCs. Moreover, hERG1 channel function proved critical for both the triggering of the prosurvival signals and the development of chemo resistance, as hERG1 channel blockers abrogated the protective effect of MSCs. These results were validated by studies in murine models of ALL.
Conclusions: Our findings indicate that hERG1 channels on leukemic cells regulate the prosurvival signals emanating from MSCs in the bone marrow microenvironment, and

suggest that administration of hERG1 blockers with conventional antileukemic agents might improve clinical outcome in patients with ALL.

ACT 21. Cell Death in Colon Cancer. Hu-r-β GBP Inhibits PI3K Activity, Overcomes PIK3CA Mutations, Induces Apoptosis and is Therapeutically Effective on Colon Caner Xenografts

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Background: Monomeric β-galactoside binding protein (β-GBP) is a newly discovered antiproliferative cytokine. In normal cells β-GBP negatively regulates the cell cycle, in cancer cells β-GBP induces apoptosis by inhibiting mitogenic and survival signaling. Mechanisms of action initiate with downregulation of class IA and class IB PI3K
Methods: Assessment of: Cell proliferation/apoptosisRas-GTP loading/ERK activation; akt gene expression/Akt activation; Rac-GTP loading/cytoskeletal actin organization
Results: In SW480, SW620 and LoVo colon carcinoma cells inihibition of PI3K activity by Hu-r-β GBP induces death through the activation of both intrinsic and extrinsic apoptosis as a consequence of cytoskeletal actin rearrangement. Analysis on 1)the effect of Hu-r-β GBP on colorectal cancer cells with deregulated PI3K activity caused by PIK3CA mutations and 2)therapeutic efficacy *in vivo* shows the following:1) HCT116 cells harbouring a H1047R mutation in exon 20 (kinase domain) and DLD cells harbouring an E545K mutation in exon 9 (helical domain) are triggered into apoptosis after inhibition of PI3K activity as colon cancer cells exempt of PIK3CA mutations2) Hu-r-β GBP strongly inhibits SW620 xenograft growth in athymic nude mice. Resumption of tumor growth after a five week period of treatment is very contained indicating clonal selection through elimination of the more aggressive cells.
Conclusions: The β-GBP molecule is a physiological PI3K inhibitor, as such it is devoid of the drawbaks of toxicity and drug resistance. Its ability to induce death in cancer cells by combined routes and its therapeutic efficacy in animal models offers a new approach with prospects in the clinic.

ACT 22. Epigenetic Modifications in a T(8;21) Acute Myeloid Leukaemia Cell Line Caused by Administration of Two HDAC Inhibitors, VPA and SAHA.

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Background: The acute myeloid leukaemia (AML) cell line Kasumi-1 is characterized by t(8;21) translocation and the consequent expression of AML1/ETO fusion protein, responsible for recruitment of histone de-acetylases (HDAC) and the consequent transcriptional repression of genes involved in myeloid maturation. We determined the effects of HDAC inhibitors (HDACi) on acetylation of histones H4 and H3, expression of Interleukin-3 (IL3) gene, target of AML1/ETO, and histone acetylation on IL3 promoter.
Methods: Kasumi-1 cells were treated with the HDACi VPA (2mM) or SAHA (1μM). Cells were lysed at different days of culture to extract proteins for Western blotting or mRNA for Real-Time PCR, or to immunoprecipitate chromatin bound to acetylated H4 or H3.
Results: VPA or SAHA increased total H4 and H3 acetylation, SAHA more rapidly than VPA. Both HDACi increased K5, K8 and K16 acetylation of H4 and K9 of H3, and SAHA also K12 of H4, in parallel with resumption of transcriptional activity. IL3 gene expression was re-induced, rapidly by SAHA, slowly but more intensely by VPA. These different effects depend on different levels of acetylated H4 and H3 on IL3 promoter. SAHA determined rapid and simultaneous acetylation of H4 in K5, K8 and K12 and of H3 in K27 residue, in keeping with its rapid but transient effects on IL3 expression. VPA induced a simultaneous acetylation of H4 in K8, K12 and K16 and of H3 in K27, compatible with its sustained effect.
Conclusions: VPA seems to be more effective than SAHA in inducing expression of gene silenced by AML1/ETO, such as IL3.

ACT 23. Proteolytic Cleavage of CD44 is Induced by the RET/PTC and BRAF Oncogenes and Sustains Autonomous Proliferation of Neoplastic Thyroid Cells.

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Background: The CD44 cell surface adhesion molecule is implicated in a variety of physiologic and pathologic processes and it is overexpressed in a wide range of cancer types, including papillary thyroid carcinoma (PTC). CD44 undergoes sequential proteolytic cleavage at the level of extracellular and intramembranous domains, by a metalloproteinase and a presenilin-dependent γ-secretase, respectively. This results in the release of a CD44 intracellular domain fragment (CD44-ICD) and a CD44 ectodomain (ectoCD44). CD44-ICD acts as a signal transduction molecule. It is translocated to the nucleus and activates gene transcription.
Methods: Expression of RET/PTC and of BRAF and RAS constitutively active mutants triggers CD44-ICD formation and chemical blockade of both RET/PTC (ZD6474) and MEK (U0126) abrogates RET/PTC-mediated CD44 cleavage. CD44 cleavage and ectoCD44 shedding occur in human PTC cells; matrix metalloproteinase (BB94) and RET/PTC (ZD6474) inhibitors reduce ectoCD44 shedding. Adoptive overexpression of CD44-ICD in normal thyroid follicular PC cells mediates TSH-independent proliferation. CD44-ICD stimulates CREB-mediated activation of CCDN1-luciferase in PC thyroid cells.
Results: Signaling of thyroid cancer-associated RET/PTC and BRAF oncoproteins induces metalloproteinase- and γ-secretase-mediated CD44 proteolysis through the ERK pathway. We demonstrate that CD44-ICD binds to CREB and enhances CREB binding to CCDN1 promoter, this results in increased CREB S133 phosphorylation and CREB-mediated transcription.Through this mechanism, CD44-ICD up-regulates cyclin D1 expression and proliferation of thyroid cells.
Conclusions: Taken together, these findings suggest that MAPK kinase pathway-mediated CD44 cleavage sustains proliferation of thyrocytes harbouring RET/PTC, RAS or BRAF oncogenes. This new pathway may provide novel levels of therapeutic intervention for thyroid carcinoma treatment.

ACT 24. A Met-Derived Peptide as Inhibitor of Angiogenesis and Vascular Tumor Growth

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Background: Hepatocyte Growth Factor (HGF) and its receptor Met are responsible for different cell responses involved in development processes and pathological conditions, including cancer. Molecules interfering with Met activity could be valuable therapeutic agents for inhibition of tumorigenic and metastatic processes triggered by Met in human cancer. Here we investigated the anti-angiogenic properties of a synthetic peptide derived from Met C-terminal tail, which was delivered into cells by fusion with the internalization sequences of two cell penetrating peptides.
Methods: *In vitro* we treated human endothelial cells with different peptides containing Met sequence fused to the internalization domain of Antennapedia or of Tat peptides and we evaluated cell proliferation. We then analyzed the ability of peptides to interfere with HGF-induced responses in endothelial cells, such as migration, invasion and morphogenesis. By western blotting analysis we investigated whether these peptides affected Met activation and downstream signaling. *In vivo* we performed matrigel sponge assay and Kaposi's sarcoma xenograft to test the peptides efficacy on angiogenesis and tumor growth.
Results: We observed that in endothelial cells peptides inhibited ligand-dependent cell proliferation, motility, invasiveness and morphogenesis in vitro, which correlated with interference of HGF-dependent downstream signaling. In vivo, the peptides inhibited HGF-induced angiogenesis in the matrigel sponge assay and significantly impaired Kaposi sarcoma xenograft tumor growth and vascularization.
Conclusions: Our results showed that the terminal sequence of Met receptor impaired angiogenesis triggered by HGF/Met interaction, suggesting the feasibility of using anti-docking site compounds as therapeutic agents to interfere with tumour progression and angiogenesis.

ACT 25. Calcium-Calmodulin Dependent Kinase II is a Site for Targeted Therapy for Treating Medullary Thyroid Carcinoma

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Background: The calcium-calmodulin dependent kinase II (CaMKII) is an ubiquitous serine/threonine protein kinase involved in multiple signalings and biological functions. This kinase participates with Ras to Raf-1 activation. The aim of this study was to characterize the state of activation of CaMKII in medullary thyroid carcinoma (MTC) bearing mutated RET and to determine the relevance of CaMKII in the aberrant proliferation of spontaneous MTC cancer cell lines.
Methods: NIH-3T3 were stably transfected with the RET mutants C634 and M918 present in the MEN 2A, MEN 2B. CaMKII activity was determined by in vitro kinase assay and Western blot. Cell proliferation was determined by cell count and (3H) thymidine incorporation assay.
Results: In the absence of stimuli, CaMKII was not activated in NIH-3T3 while it was strongly activated in cell mutants expressing mutated RET. We then analyzed two cell lines originated from spontaneous sporadic MTC. Both TT and RO-H85-1 (bearing C638 and M918 mutation respectively) were analyzed for CaMKII state of activation and were treated with the CaMKII inhibitors KN93 or antCaNtide or transfected with the dominant negative CaMKII K42M. CaMKII was expressed in the two cell lines at comparable levels. CaMKII was constitutively activated by an up-stream calcium-calmodulin dependent signal. The inhibition of this signal or inhibition of CaMKII reduced the cell proliferation and induced cell death
Conclusions: CaMKII is constitutively activated in MTC by the mutated RET and its inhibition impairs cell proliferation. These results support the potential application of CaMKII inhibitors in the treatment of MTC.

ACT 26. ZD6474 Inhibits Growth and Tumorigenicity of RET-Mutation Positive Medullary Thyroid Carcinoma Cells

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Background: Medullary thyroid carcinoma (MTC) is a neuroendocrine tumor of thyroid C-cells. MTC occur as sporadic tumors in almost 80% of cases, the remainder are affected by one of three autosomal dominant familial cancer syndromes, MEN2A, MEN2B and FMTC. Treatment of sporadic MTC generally consists of a total thyroidectomy; both chemotherapy and radiotherapy have had only limited effectiveness in MTC. MTC are often caused by gain of function of the RET tyrosine kinase receptor coding gene. ZD6474 (vandetanib) is a ATP-competitive inhibitor of RET, EGFR and VEGFRs kinases.
Methods: We have studied ZD6474 mechanism of action in TT and MZ-CRC-1 human MTC cell lines, carrying the cysteine 634 to tryptophan (C634W) and the methionine 918 to threonine (M918T) RET mutation, respectively.
Results: ZD6474 caused *in vitro* growth arrest and inhibited RET, Shc and p44/p42 MAPK phosphorylation in these cell lines. It also significantly reduced the size of TT tumor xenografts in nude mice, which is associated with reduced blood vessel counts and phospho-RET suppression. Knockdown of RET, EGFR or KDR by RNA interference showed that MTC cells depended on RET for their proliferation. In fact the expression of the ZD6474-resistant V804M RET mutant rescued proliferation of TT cells under ZD6474 treatment, showing that RET is a key ZD6474 target in these MTC cells. However, upon RET inhibition, stimulation of EGFR was able to partially rescue TT cell proliferation and downstream pathway.
Conclusions: Simultaneous inhibition of RET and EGFR by ZD6474 may overcome the risk of MTC escape from RET blockade.

ACT 27. Natural Flavonoids Inhibit Proliferation and Promote Differentiation of Colon Cancer Cells

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Background: Flavonoids are polyphenolic compounds that are ubiquitous in plants and to which we are all exposed consuming foods of plant origin.
Methods: This study was designed to evaluate and compare the *in vitro* effects of naringin and naringenin, two of the most abundant Citrus bioflavonoids, and of silybin, a flavonoid derived from milk thistle (Silybum marianum).
Results: The three compounds were evaluated on two colon cancer cell lines (HT29 and CT26) and were able to inhibit cell proliferation in a time- and dose-dependent fashion. These effects were associated with an inhibition of cell cycle progression and accumulation of cells in the G0/G1 phase of the cell cycle, although with some differences amongst the three compounds. Western blot analyses displayed significant changes in the expression levels of cell cycle regulators with a decrease of cyclin D1 and an increase of p27 and p21 being the most frequent alterations. No changes were observed in the expression levels of cyclin E and cyclin A. An interesting finding was that silibinin and, to a greater extent, naringin were able to promote sodium butyrate-induced differentiation of CaCo2 cells and the associated reduction in the expression of the stem cell marker CD133. Since clinical applications of polyphenols are hampered by their low solubility in water we have also developed more soluble derivatives which are currently under analysis.
Conclusions: Overall, our findings demonstrate the ability of these compounds to inhibit proliferation and promote differentiation of colon cancer cells and warrant further studies to evaluate their suitability as chemopreventive agents.

ACT 28. Hepatocellular Carcinoma Cells with Mutated β-Catenin are Glutamine Addicted: Targeting Amino Acid Metabolism for a Novel Therapeutic Approach

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Background: Glutamine Synthetase (GS) is a marker of β-catenin mutated Human Hepatocellular Carcinomas (HCC), suggesting that glutamine has a central role in the metabolism of these tumors. To verify this hypothesis, we tested glutamine dependency of wild type (w/t) or β-catenin-mutated HCC cell lines.
Methods: Three w/t β-catenin HCC cell lines (Huh-7, Hep3B, PLC/PRF/5) and the β-catenin mutant line HepG2 were treated with the glutaminolytic drug L-Asparaginase and the GS inhibitor Methionine-L-Sulfoximine (MSO) to achieve glutamine depletion. Cytotoxic effects were assessed with resazurin viability assay and cytofluorimetry. Cell amino acids were analyzed through HPLC, gene expression and phospho-proteins abundance with qPCR and immunoblots, GS and caspase-3 activity through biochemical assays.
Results: Under control conditions HepG2 cells showed the highest glutamine content, as well as high expression of GS and of the amino acid transporter SNAT2. L-Asparaginase alone caused a dramatic drop of cell glutamine content in all the lines, but had significant effects on proliferation, cell cycle, p21waf1, caspase-3 activity, and apoptosis only in HepG2 cells. Consistently, the phosphorylation of eIF2α and the inhibition of Akt and mTOR were more pronounced in HepG2 than in Huh-7 cells. Moreover, in HepG2 cells the combined treatment with L-Asparaginase and MSO stimulated dramatically Akt and mTOR activity, suggesting a perturbing effect of the GS inhibitor on cell nutrient sensors.
Conclusions: Thus, compared to cells with a wild type β-catenin, HepG2 cells are more sensitive to glutaminolytic drugs and GS inhibitors, indicating that they are glutamine-addicted.

ACT 29. Structure-Activity Studies of CaMKIINtide Peptide Analogs and Potential Anti-Proliferative Role

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Background: Ca2+/calmodulin-dependent protein kinase II (CaM-KII) is a major mediator of cellular Ca2+ signaling, that plays an important role in many cellular function including cell division, differentiation, cardiac contraction, and synaptic plasticity.1 Several inhibitors are commonly used to study CaMKII function, but these inhibitors all lack specificity. The natural CaMKII inhibitor protein, CaM-KIIN, provides a promising alternative, because it potently inhibits CaMKII but not CaMKI, CaMKIV, PKA or PKC. COOH-terminal truncations of CaM-KIIN indicated that its inhibitory potency and activities resided largely in a 27 aminoacid residues, named CaM-KNtide. The high specificity of this peptide makes it a very useful probe for identifying specific CaMK-KII reactions
Methods: CaMKII activity was tested on Autocamtide in the presence of the different peptides. Autocamtide is a highly selective synthetic peptide substrate for Ca2+/Calmodulin-Dependent Protein Kinase II (CaMKII) derived from the autophosphorylation site of the a subunit of CaMKII.CaMKIINtide peptide analogs were synthetized by convetional Fmoc-based solid-phase strategy in a manual reaction vessel, using Wang resin.
Results: We synthetized seven peptides that cover the entire CaM-KNtide sequence, each peptide had sequences shifts of five residues, from both NH2 and COOH terminal. In addition, a Cell Permeant Sequence (TAT) was added to the N-terminal of CaMKNtide, to make it cell-permeable. We tested all the synthetized peptides *in vivo* and *in vitro* CaMKII kinase assay.
Conclusions: We identified the minimal inhibitory region of CaMKIINtide sequence, this peptide strongly inhibits CaMKII activity and has no effect on CaMKI and CaMKIV activity.

ACT 30. Ca2+/Calmodulin-Dependent Protein Kinases II and IV Oppose Each Other in the Regulation of Leukemia Cell Proliferation

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Background: The Ca2+/calmodulin (CaM) dependent protein kinases (CaMKs) link transient increases in intracellular Ca2+ with different physiological processes. CaMKII is a ubiquitous enzyme that regulates cell proliferation and cell cycle progression. CaMKIV is a tissue-specific enzyme that mediates cell survival and differentiation. The two enzymes present some overlapping targets but play different roles in biological functions.
Methods: U-937 cells were cultured RPMI-1640 Medium.Lentivirus-green fluorescent protein (GFP) constructs were generated and characterized according to Kokubo, M (2009) J Neurosci 29, 8901-8913. DNA synthesis was determined by [3H]thymidine incorporation assay. Dual Luciferase Assay was permormed using a dual Luciferase Assay Reporter System Kit (Promega Co., Madison, WI). QRT-PCR for CaMK4 was performed using a Bio-Rad IC5 thermo cycler.
Results: CaMKIV expression is up-regulated following inhibition of CaMKII using the pharmacological inhibitor KN93 and/or the CaMKII-specific inhibitory peptide AntCaNtide. In transient transactivation assays, CaMKII represses retinoic acid receptor β (RARβ)-mediated activation of the CaMK4 promoter. Contrarily, ectopic expression of wild type CaMKIV in U937 cells inhibits the activation of CaMKII. Furthermore, CaMKIV expression inhibits U937 cell proliferation and cell cycle progression: cell functions regulated by CaMKII. U937 cells expressing CaMKIV a marked reduction in DNA synthesis and undergo a G0-G1 phase arrest.
Conclusions: Together, our data suggest that the balance between CaMKII and CaMKIV regulate cell cycle: the prevalence of CaMKII leading to cell cycle progression and proliferation, whereas CaMKIV promotes a state of quiescence. Our novel finding of a reciprocal regulation between the two CaMKs provides basis for development of therapeutic tools in the control of proliferative disorders.

ACT 31. Calcium/ Calmodulin- Dependent Kinase IV Expression Regulates Cell Proliferation in Colon Carcinoma

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Background: The multifunctional CaMKs (CaMKI, II, IV) link transient increases in intracellular Ca2+ with physiological process such as proliferation, differentiation and survival. CaMKII is a ubiquitous enzyme that promotes cell proliferation by activating the ERK pathway. CaMKIV is a Ser/Thr enzyme expressed predominantly in brain, thymus, testis, ovary, bone marrow and hematopoietic system, and it mediates cell survival and differentiation in a number of cell types.
Methods: Protein levels were determined by WB. DNA synthesis was performed by [3H]thymidine incorporation assays.
Results: We examined CaMKIV expression in KAT-4 HT-29 cell lines, and found low expression of CaMKIV . The re-expression of CaMKIV led to a reduction in cell proliferation and DNA synthesis. In CaMKIV expressing cells CaMKII activation by ionomicin is inhibited with a corresponding suppression of ERK phosphorylation. We analysed the levels of the downstream targets of CaMKIV, as well as of some pro- and anti-apoptotic proteins. CaMKIV expressing cells presented a marked increase in CREB phosphorylation, whereas Bcl-2 expression did not significantly change. Furthermore, CaMKIV expression in KAT-4 and HT-29 led to a marked increase in the expression of the pro-apoptotic protein Bax and to a reduction of phosphorylated Bad.
Conclusions: Our data suggest that: i)CaMKIV regulates the abnormal proliferation of colon-cancer cell lines, and ii) the inhibition of CaMKII plays a role in this process. These findings have potential therapeutic implications, due to the pro-mitogenic activation of CaMKII in a number of cancers.

ACT 32. Docosahexaenoic Acid Reverts the Resistance to UV-Induced Apoptosis by Inhibiting HuR-Dependent COX-2 mRNA Stabilization in Human Keratinocytes

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Background: n-3 PUFAs have shown increasing interest as factors potentially able to inhibit the carcinogenic activity of UV radiation (UVR). Dysregulation of apoptosis is crucial to explain the carcinogenic effects of UVR, removing damaged premalignant cells after an acute irradiation while apoptotic resistance and carcinogenesis may be induced by the chronic UVR exposure of skin cells.Our aim was to study the possible action of DHA in the prevention of apoptosis resistance to UVR in a keratinocyte cell line, and identify the molecular mechanism of its action.
Methods: Artificial acute irradiation with UVA and UVB was performed. Apoptosis was evaluated by acridine orange/propidium iodide method and immunofluorimetrically with M30 antibody. Cytokeratins, HuR and COX proteins were evaluated by Western Blotting. Caspase-3 activity was measured by fluorimetric assay and COX-2 mRNA by RT-PCR analysis.
Results: By serially sub-culturing the immortalized keratinocytes HaCaT cells we obtained a new cell line (nr-HaCaT) resistant to UV-induced apoptosis. These cells showed lower differentiation than the parental cells and COX-2 overexpression, considered an early marker of carcinogenesis in various tissues, including skin. Treatment with 10-50 μM DHA reverted their resistance to UV-induced apoptosis, increasing the Bax/Bcl-2 ratio and caspase-3 activity. DHA reduced COX-2 expression through the inhibition of COX-2 mRNA stabilization induced by HuR. The results obtained by transfecting nr-HaCaT cells with HuR siRNA further enforced our hypothesis.
Conclusions: Our findings support the role of DHA as an anticarcinogenic factor against non-melanoma skin cancers and elucidate a possible molecular mechanism of action.

ACT 33. Novel Regulatory Mechanism of Aromatase Activity in Breast Cancer

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Background: Estrogens are strongly associated with breast cancer progression. The intratumoral conversion of androgens to estrogens by aromatase within the breast is an important mechanism of autocrine stimulation in hormone-dependent breast cancer.
Methods: Aromatase activity was evaluated by tritiated water release assay. Western blotting and immunoprecipitation analisys, along with *in vivo* labeling and site-directed mutagenesis studies were performed to evaluate phosphorylation status of aromatase protein and the residues involved.
Results: We show that 17-β estradiol (E2) rapidly enhances aromatase enzymatic activity through an increase of aromatase phosphorylation in breast cancer cells. Phosphorylation of the 361 tyrosine residue is crucial in the up-regulation of aromatase activity under E2 exposure. We demonstrated a direct involvement of non-receptor tyrosine-kinase c-Src in E2-stimulated aromatase activity since inhibition of its signalling abrogated the up-regulatory effects induced by E2 on aromatase activity as well as phosphorylation of aromatase protein. In addition, it emerges that aromatase is a target of crosstalk between growth factor receptors and estrogen receptor a signaling.
Conclusions: These findings show that tyrosine phosphorylation processes play a key role in the rapid changes induced by E2 in aromatase activity revealing a short non genomic autocrine loop between E2 and aromatase in breast cancer cells.

ADVANCES IN MOLECULAR THERAPIES

AMT 01. Cannabinoid Type-1 Receptor Signaling Modulates Human Sperm Physiology: Implications for Fertility

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Background: Accumulating evidences have suggested a key role for the endogenous cannabinoid (EC) system in reproduction and recently it was also identified in spermatozoa. By transmission electron microscopy we have demonstrated that the cannabinoid type-1 receptor (CB1-R) was located in the human sperm membrane of the head and at mitochondrial level, suggesting its potential role in the modulation of sperm fertilization and metabolism. To elucidate the EC system role and in particular of CB1-R in male fertility, we investigated the effects of CB1-R agonism and antagonism on fertilizing ability and energy metabolism of human sperm.
Methods: We analyzed *in vitro* the effects of the CB1-R agonism and antagonism on capacitation process and evaluated lipid and glucose metabolism in human sperm samples.
Results: Agonism at CB1-R, either by exogenously administered anandamide or by increased endogenous EC tone determined negative effects on sperm motility, survival and capacitation and had a lipogenic and glucogenetic effect. Besides anandamide was able to regulate insulin secretion, a marker of the capacitation process. Interestingly, the blockage of the CB1-R improved all sperm activities including acrosome reaction and induced energy expenditure.
Conclusions: The EC system has been recognized as a crucial player in energy balance control. Similarly, in humans its modulation seems to regulate sperm energy homeostasis, therefore the CB1-R blockage could increase human sperm fertilizing ability through promoting energy expenditure to be used during capacitation and acrosome reaction. Our data support the hypothesis that pharmacological inhibition of the CB1-R might be used to develop special media to improve *in vitro* fertilization.

AMT 02. Downregulation of uPAR Expression and Inhibition of Retinal Neoangiogenesis by an Antisense Oligonucleotide Targeting uPAR mRNA in a Mouse Model of Proliferative Retinopathy

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Background: The main pathogenetic event of severe retinal diseases known as proliferative retinopathies, from age related macular degeneration to diabetic retinopathy, is neoangiogenesis. This hypoxia-induced process, by which new blood vessels are formed by the sprouting of endothelial cells from preexisting vessels, results from upregulation of specific proteases, including urokinase (uPA) and its receptor (uPAR), which induces matrix remodeling and cell migration. On the basis of our previous results and evidences reported in the literature, we have hypothesized that uPAR knocking down by antisense oligonucleotides (ODNs) could reduce retinal vessel production. We have verified this hypothesis both *in vitro* and *in vivo*.
Methods: Both human retinal endothelial cells (hREC) and a mouse model of neoangiogenesis-related retinopathy (premature retinopathy) have been used. uPAR knocking down was obtained by a chemically stabilized anti-uPAR aODN added to the culture medium or injected intraperitoneally (IP) for the *in vitro* or *in vivo* experiments, respectively. The molecular and phenotypic effects of the anti-uPAR aODN were than evaluated.
Results: The anti-uPAR aODN (10 μM) markedly inhibited uPAR expression in cultured hREC cells and lowered capillary morphogenesis and cell migration. When applied IP to the retinopathy mouse model, the aODN reached the retina, where it lowered uPAR expression, thereby markedly increasing the retina avascular areas and reducing the retinopathy score.
Conclusions: We are evaluating this aODN as topical therapeutic for proliferative retinopathies. Acknowledgements to ECR Firenze, FCR Lucca, ASI for funding this research

AMT 03. Control of Rheumatoid Arthritis Synovial Fibroblasts Cartilage Invasion by Loss of Function of the Cell Fibrinolytic System *In Vitro* and *In Vivo*

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Background: In rheumatoid arthritis (RA) the mechanisms of synovial membrane proliferation and invasion of the underlying cartilage are still poorly understood. The

urokinase-type plasminogen activator (uPA), the uPA receptor (uPAR), and plasminogen activator inhibitor-type 1 (PAI-1) are pivotal in proliferation and invasion of several cell types, including synovial fibroblasts (SF). We undertook this study to investigate the possibility to control RA-SF invasion *in vitro* and *in vivo* by inhibition of uPA and uPAR.
Methods: uPA, uPAR and PAI-1 were measured by ELISA and by RT-PCR of mRNAs. uPA activity was also studied by zymography. Proliferation was measured by cell counting, cell invasion with the Boyden chamber assay and by quantification of cells passaging through Matrigel-coated porous filters. The "inverse wrap" human cartilage and RA-SF implantation in the SCID mouse model for RA was used to study cartilage invasion *in vivo*.
Results: RA-SF over-expressed uPAR and PAI-1 and were more prone than their normal counterpart to Matrigel invasion and to proliferation. This effect was counteracted by an active site inhibitor of uPA enzymatic activity (WX-340) and by uPAR antisense treatment. The use of both compounds *in vitro* showed cooperative effects which were more intense than the single components in RA-SF. Significant inhibition of cartilage invasion was obtained *in vivo* with uPAR antisense treatment, while uPA inhibition was inefficient either alone or in combination with the antisense treatment.
Conclusions: The reduction of uPAR expression in RA-SF reduces invasion of human cartilage *in vitro* and in the SCID mouse model.

AMT 04. Effect of Polyethylenimine on Lentiviral-Mediated Transduction of Airway Epithelial Cells

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Background: Lentiviral vectors (LV) are promising agents for efficient gene therapy of genetically determined pulmonary diseases, such as cystic fibrosis, in which the target is the respiratory epithelium. However, LV have not been evaluated for cytotoxicity and effect on the tightness of the airway epithelium.
Methods: We evaluated the transduction efficiency of LV expressing Green Fluorescent Protein (GFP) gene as well as cytotoxicity and tight junction (TJ) integrity in a model of polarized bronchial epithelial cells (16HBE14o-).
Results: High multiplicities of infection (MOI, up to 2000) resulted in up to 65% of GFP-positive cells, but they were cytotoxic, as assessed by increase in propidium iodide staining, and harmful for the epithelial tightness, as demonstrated by the decrease of transepithelial resistance (TER) and delocalization of occludin from the TJs. To increase LV efficiency at low LV:cell ratio, we employed non-covalent association with the polycation branched 25 kDa polyethylenimine (PEI). Transduction of cells with PEI/LV particles resulted in 2.5-3.6 fold increase of percentage of GFP positive cells by using 1 X 10^7 PEI molecules/transducing units with 50 MOI LV as compared to plain LV. At this dose PEI/LV transduction resulted in 6.5 ± 2.4% of propidium iodide-positive cells. On the other hand, PEI/LV particles did not determine any alteration of TER and TJ organization.
Conclusions: We conclude that PEI can facilitate LV-mediated transduction of airway epithelial cells in the absence of overt cytotoxicity and alteration in epithelial tightness. PEI/LV deserves further evaluation as a novel gene transfer agent for cystic fibrosis.

AMT 05. Local Disregulation of Calcium and Phosphate Balance in the Pathogenesis of Ectopic Calcification

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Background: Ectopic calcification occurs as a consequence of necrosis or of augmented circulating calcium and phosphate, but also in the presence of abnormal metabolic factors controlling mesenchymal cell activity. Within this context, Pseudoxanthoma elasticum (PXE), a genetic connective tissue disease characterized by progressive mineralization of elastic fibres, represents a very informative experimental model since calcifications are associated with mutations of the ABCC6 gene (belonging to the ABC transporter family) in the absence of cell death and of increased calcemia and phosphatemia.
Methods: Parameters of calcium and phosphate homeostasis have been investigated by RT-PCR and Western blot in cultured dermal fibroblasts.
Results: In PXE fibroblasts several calcification markers (i.e. ENPP1, ANKH, CA II, BMP-2 and 7), are expressed without significant differences compared to control cells. Interestingly, there was a significant upregulation of TNAP (tissue non-specific alkaline phosphatase), a phenotypic marker of osteoblasts, that increases phosphate availability and reduces the levels of pyrophosphate, a mineralization inhibitor. At the same time, calcium deposition cannot be adequately regulated by other inhibitors as MGP (matrix Gla Protein). This protein is not properly activated by a vitamin K-dependent carboxylation process due to abnormal expression of proteins of the endoplasmic reticulum as calumenin, an inhibitor of gamma-carboxylase, and protein disulfide isomerase, that is involved in vitamin K recycling by interacting with VKOR and Ero-1.
Conclusions: This study demonstrates that calcification of soft connective tissues may occur when calcium and phosphate homeostasis are modified at local levels, thus opening new perspectives for therapeutic approaches.

AMT 06. Evaluation of Polyaminoacidic Polymers as Gene Transfer Agents Into Respiratory Epithelial Cells and of Their Biophysical Properties in the Presence of Cystic Fibrosis Sputum

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Background: Cystic Fibrosis (CF) is caused by mutations in the CFTR (CF Transmembrane Conductance Regulator) gene, which results in the accumulation of sticky mucus on the epithelial surfaces of many organs, including the lung. Non viral vector-mediated gene transfer into airway epithelial cells is a gene therapy strategy actively pursued in CF. However, since low efficiency, due to the airway mucus, does not presently grant a therapeutic effect, it is rational to search for and test new non-viral vectors.
Methods: Here we examined two gene delivery formulations based on the use of novel polyaminoacidic polymers derived from α,β-poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA), i.e. PHEA-spermin (Spm) and PHEA-PEG-Spm, and their biophysical properties in the presence of CF sputum.
Results: Both polyplexes mediated luciferase gene expression in immortalized respiratory epithelial cells with DNA/polymer mass ratio dependency. However, transfection efficiency was 2-log less when polyplexes were formed in water. Interestingly, in water, both polyplexes showed a significant cytotoxicity at highest copolymer/DNA mass ratios. Both copolymers condensed plasmidic DNA in a mass ratio dependent fashion and their size and charge (zeta-potential) was strongly influenced by solvent and polymer/DNA mass ratio. Fluorimetric analysis showed that the CF sputum (pooled from 20 patients) disassembled copolymer polyplexes, with PHEA-PEG-Spm appearing more stable than PHEA-Spm.
Conclusions: These results indicate the usefulness of PHEA copolymers in mediating transfection of airway epithelial cells, although a suitable formulation with low cytotoxicity for in vivo experiments has to be found, and suggest that PEGylation may increase their stability and efficiency in CF-like conditions.

AMT 07. Combined Antitumoral Efficacy of the Histone Deacetilase Inhibitor LBH589 and the Lipid Peroxidation End Product 4-Hydroxinonenal (HNE) on PC3 Prostate Cancer Cell

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Background: Epigenetic regulations are commonly altered in tumors and consequently histone deacetylase inhibitors (HDACi) are become promising antineoplastic agents. In particular, LBH589 (Novartis) is a potent pan DAC inhibitor, with antineoplastic effects. 4-Hydroxynonenal (HNE) is an aldehyde showing antiproliferative and prodifferentiative effects on varies tumor cells. Previously we demonstrated that HNE affected the antitumoral efficacy of the HDACi, LBH589 in the PC3 prostatic cancer cells as a consequence of a relevant block in G2 and M phase of cell cycle, followed by apoptosis.
Methods: We investigated histone H3 acetylation status and microarray gene expression profiling in PC3 cells treated with HNE or LBH or the drugs in combination.
Results: A greater and prolonged increase of H3 acetylation in cells treated with the combination has been observed. Furthermore, FACS analysis showed that the combined treatment not modify the acetylation status of H3, but increases the number of cells presenting hyperacetylation. Microarray gene expression profiling revealed in the combined treatment a dramatic change in gene expression, involving genes such as MCM4 (Minichromosome Maintainance Complex 4), HMOX1 (Heme Oxigenase Decycling1), CDKN1A (P21). These modulations have been validated by Real Time PCR also.
Conclusions: Our results demonstrated that HNE increases LBH589 activity and they can provide new insight in the interaction between HDACi and lipid peroxidation products.

AMT 08. Targeting Cell Cycle Through Combination of the Histone Deacetylase Inhibitor, LBH589, and the Lipid Peroxidation End Product, 4-Hydroxynonenal (HNE), in PC3 Prostate Cancer Cells.

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Background: LBH589 (Novartis) is a potent Histone Deacetylase Inhibitor (HDACi) currently under investigation for the treatment of prostate cancer. Indeed, LBH589 can act as a strong radiosenzsitizing agent and this combinational strategy is currently undergoing in clinical trials.Radiation therapy increases oxidative stress and lipid peroxidation of PUFA. HNE, an aldheydic compound derived from lipoperoxidation is considered a second end messenger of oxidative stress. It inhibited proliferation and induced apoptosis in several tumor cells.
Methods: In the present study we tested if HNE could potentiate the antititumoral efficacy of LBH589 in PC3 cells through colony forming assay and cell cycle studies.

Results: Both HNE and LBH inhibited cell proliferation, however the combination of the two agents induced a synergistic effect. Cell cycle analysis showed a relevant arrest in G2/M phase, followed by cell death.The nature of the cell cycle arrest was different: LBH589 induced prevalently a G2 arrest whereas HNE induced a mitotic block at the metaphase level. The combined treatment showed a greater mitotic block respect to the treatment with HNE only. Gene expression analysis revealed a complex scenario of modulation of different cell cycle regulators.
Conclusions: In conclusion we demonstrated that HNE potentates the antitumoral activity of LBH589 through cell cycle regulations resulting in cell death.

AMT 09. Globoid Cell Leukodystrophy: Inhibition of Angiogenesis by β-Galactosylceramidase Deficiency

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Background: Globoid cell leukodystrophy (GLD), or Krabbe's disease, is a neurological disorder caused by genetic β-galactosylceramidase (β-GAL) deficiency. This results in the accumulation of the neurotoxic metabolite psychosine in the central nervous system (CNS) paralleled by oligodendrocyte apoptosis, demyelination, and formation of microglial globoid cells. However, neovascularization plays an important role in CNS development. Here, we addressed the possibility that alterations in blood vessel development may contribute to the CNS damage that occurs in Krabbe's disease.
Methods: Psychosine was tested for its capacity to affect angiogenesis in different *in vitro*, *ex-vivo*, and *in vivo* assays. Also, the neovascularization process was investigated in Twitcher mice, a genetically and enzymatically authentic mouse model of human GLD.
Results: We have found that endothelial cells (ECs) express b-GAL and that psychosine has antiangiogenic properties *in vitro*, *ex-vivo* and *in vivo* by causing the disassembling of EC actin structures. Accordingly, histological and ultrastructural defects were observed in the brain vasculature of Twitcher mice. Also, ECs from Twitcher mice show a remarkable impairment in their capacity to mount a neovascular response when challenged *in vivo* and *ex-vivo* by angiogenic growth factors.
Conclusions: Taken together, these data point to a previously unrecognised role of β-GAL in EC biology and angiogenesis. At present, we have cloned two β-GAL orthologues in Zebrafish (Danio rerio). Studies are in progress to assess the spatial and temporal expression of β-GALs in Zebrafish embryos and the effect of morpholino-mediated b-GAL knock-down on Zebrafish development.

AMT 10. 5-Fluorouracil-induced Cardiovascular Toxicity

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Background: Therapy with 5-FU and with capecitabine is used in treatment of solid tumors such as gastrointestinal (esophageal, gastric, pancreatic, colorectal and hepatocellular), gynecological, head and neck, and breast carcinomas. Therapy with 5-FU can have adverse effects, especially an occasional life-threating cardiac toxicity. Signs of 5-FU-induced cardiotoxicity include angina pectoris and acute myocardial infarction, less frequently arrhythmias, heart failure, and cardiogenic shock. The mechanisms of 5-FU-induced cardiotoxicity are not well defined. The aim of this study is to define the effects of 5-FU on cardiomyocytes and endothelial cells.
Methods: We tested the effects of 5-FU on primary cell cultures of human cardiomyocytes and human umbelical vein endothelial cells (HUVEC). We performed MTT and Sulphorodamine assay and the induction of apoptosis by flow cytometry. Cell cycle was also analysed. The disruption of mitochondrial transmembrane potential (ΔΨm) and citoplasmic accumulation of reactive oxygen species (ROS) were tested by fluorescent probes.
Results: Addition of 5-FU to endothelial and cardiac cells showed that the treatment induced a apoptosis in a time/dose-dependent manner in both cell types. Low levels of disruption in the mitochondrial transmembrane potential and low ROS accumulation were observed.
Conclusions: Our results support the hypothesis that 5-FU acts directly on the vascular compartment of the cardiac system inducing apoptotic cell death in endothelial cells. This effect is probably an aspect of a more complex mechanism, thus further studies are needed to clarify this issue.

AMT 11. The Regulatory Role of Mesenchymal Stem Cells on Immune Effector Functions

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Background: Mesenchymal stem cells (MSCs) can differentiate into bone, cartilage, muscle, fat, and other connective tissues. In addition, MSCs may also promote angiogenesis and modulate immune reactivities. The most dramatic effect of MSCs in clinical application is the control of GvHD. The molecular mechanism of MSCs modulating immune response is still poorly understood.
Methods: We examined the proliferation potential and surface marker expression of MSCs derived from BM of different ages of donors (12 to 55 yr), analyzed hematopoietic receptor/migration surface markers, characterized chemokine/cytokine production when MSCs were cocultured with autologous or allogeneic PHA-stimulated PBMCs, and examined effects of MSCs on antigen-specific T cell response.
Results: There is no correlation between the age of donor and the proliferation potential of the MSCs. We detected constitutive expression of CD73, CD29, CD90, and CD105, but not CD45, HLA-DPDQDR, CD14, CD34, and CD117 and MSCs expressed increased levels of mucosal, gut and BM homing markers. In autologous MSC/PBMC coculture, we detected reduced IL-1b and TNFα but increased IL-6, IL-8, IL-10, IP-10, MIG and RANTES expression, whereas in allogeneic settings, all of these chemokines/cytokings were increased. The effect of MSCs on the proliferation of activated PBMCs was donor-dependent, suggesting that HLAs may play a role. When MSCs were added, the IFNg/TNFα effector functions of both CD4 and CD8 T cells were down-regulated.
Conclusions: We found that MSCs modulate immune response by affecting both effector and tissue homing functions of the T cells. In addition, clinical applications of allogeneic MSCs may still be affected by HLA disparity.

CELL DEATH PATHWAYS

CDP 01. Trail is Involved in Hypoxia-Induced Apoptosis of Acute Myeloid Leukemia T(8;21)-Positive Cells.

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Background: 15-20% of acute myeloid leukemia (AML) are caused by the chromosomal aberrations inv(16) or t(8;21), the latter determining the expression of the AML1/ETO fusion protein, responsible for recruitment of histone de-acetylases (HDAC) and the consequent transcriptional repression of genes involved in myeloid maturation. We determined the effects of severe hypoxia on t(8;21) AML cells as for AML1/ETO expression, histone acetylation, proliferation, apoptosis and involvement of TRAIL therein.
Methods: We used Kasumi-1 t(8;21)-positive cells and U937-A/E cells where ectopic AML1/ETO expression is inducible. Cells were incubated at 0.3% O2 or normoxia and treated with blocking anti-TRAIL antibodies or control antibodies of the same isotype. At different days, cells were counted, stained with propidium-iodide, subjected to annexin-V test or lysed for western blotting or real-time PCR.
Results: Hypoxia reduced viability and proliferation of Kasumi-1 cells and increased apoptosis, confirmed by activation of caspases 9, 8, 3. Hypoxia also induced AML1/ETO suppression and histone H4 acetylation. Using U937-A/E cells, we found that hypoxia induced high apoptosis levels only in AML1/ETO-expressing cells, underlining the relationship between hypoxia as apoptotic agent and AML1/ETO as sensitizer to apoptosis. AML1/ETO induced in U937-A/E cells mRNA expression of TRAIL and its receptor DR5, to levels similar to those of Kasumi-1 cells. Hypoxia maintained TRAIL mRNA stable, but markedly induced TRAIL protein. Anti-TRAIL blocking antibodies significantly reduced hypoxia-induced apoptosis of Kasumi-1 cells and of AML1/ETO-expressing U937-A/E cells.
Conclusions: Hypoxia seems to play an anti-leukemic effect and work as epigenetic modulator. The high sensitivity of AML1/ETO-positive cells to hypoxia-induced apoptosis involves TRAIL.

CDP 02. Modulatory Effects of Eicosapentaenoic Acid (EPA) and Arachidonic Acid (ARA) on Cell Growth of HepG2, a Human Hepatoma Cell Line

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Background: n-3 and n-6 polyunsaturated fatty acids (PUFA) have effects on diverse processes such as cancer. We investigated the effects of eicosapentaenoic acid (EPA) and arachidonic acid (ARA) on the proliferation and apoptosis of human hepatoma cell line HepG2 after exposure to increasing concentrations of EPA or ARA for 48 h. The gene expression of Fatty Acid Synthase (FAS) and 3-Hydroxy-3-Methyl-Glutaryl Coenzyme A Reductase (HMG-CoAR) was also investigated.
Methods: Cell growth and apoptosis were assayed by MTT method and ELISA test (Roche Diagnostic, Mannheim, Germany) respectively, after cell treatment with EPA or ARA (1, 10, 30, 50, 80 and 100 μM). RT real-time PCR was used to detect FAS and HMG-CoAR mRNA levels in treated cells.
Results: EPA inhibits HepG2 cell growth in a dose-dependent manner, starting from 30 μM ($P<0.01$, one-way ANOVA test and Dunnet's post test) and exerts a significant pro-apoptotic effect already at 1 μM of EPA. An higher dose of ARA determined a significant cell growth inhibition and pro-apoptotic effect in same cells (100 μM, $P<0.01$, one-way ANOVA test and Dunnet's post test). A down-regulation of FAS and HMG-CoA mRNA was observed after EPA and ARA treatment in HepG2 cells, starting at 10 μM ($P<0.05$, one-way ANOVA test and Dunnet's post test).
Conclusions: Our results demonstrate that EPA and ARA inhibit HepG2 cell proliferation through a pro-apoptotic effect. The down-regulation of FAS and HMG-CoAR gene

expression by EPA and ARA might be one of the mechanisms for the anti-proliferative properties of PUFA in hepatocellular carcinoma.

CDP 03. Upregulation of LDL-R and CB1-R Gene Expression in HepG2 Cell Line After Treatment with Eicosapentaenoic Acid (EPA)

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Background: Dietary n-3 polyunsaturated fatty acids have effects on processes impacting normal health and chronic disease, such as cancer. Eicosapentaenoic acid (EPA) has been demonstrated to induce apoptosis and cell cycle arrest in various cancer cell lines in vitro. Aim. We investigated the anti-proliferative and pro-apoptotic effects of EPA on hepatoma cell line HepG2 by investigating the gene expression of Low Density Lipoprotein Receptor (LDL-R) and Cannabinoid Receptor 1 (CB1-R), both cell growth negative regulatory receptors.
Methods: HepG2 was treated with increasing concentrations of EPA (1, 10, 30, 50, 80 and 100 μM) for 48 hours. MTT assay was used to evaluate the cell growth. Apoptosis was evaluated by ELISA test (Roche Diagnostic, Mannheim, Germany). Reverse-transcription and real-time PCR was used to detect LDL-R and CB1-R mRNA levels.
Results: EPA reduced HepG2 cell proliferation in a dose-dependent manner. This anti-proliferative effect was significant at 30 μM of EPA and up to 100 μM ($P<0.01$, one-way ANOVA test and Dunnet's post test). A significant pro-apoptotic effect was observed already at 1 μM of EPA ($P<0.01$, one-way ANOVA test and Dunnet's post test). Moreover, EPA up-regulated the expression of LDL-R and CB1-R mRNA in HepG2 cells in a dose-dependent manner, starting at 80 μM ($P<0.05$, one-way ANOVA test and Dunnet's post test).
Conclusions: EPA inhibits the growth of HepG2 cells and mediates its effect via a pro-apoptotic mechanism. It appears that the effects of EPA on these cells are determined by the induction of LDL-R and CB1-R mRNA.

CDP 04. Upregulation of Inducible Nitric Oxide Synthase (iNOS) and Poly (ADP-ribose) Polymerase-1 (PARP-1) in Human Atherosclerotic Aorta

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Background: Reactive oxygen and nitrogen species produced in the vascular environment play a major role in the pathogenesis of atherosclerosis. Among the downstream effects triggered by oxidants, the activation of poly (ADP-ribose) Polymerase-1 (PARP-1), a DNA-repair enzyme activated in response to DNA damage, has been recently implicated in the pathogenesis of some diseases. Although, PARP-1 expression has been demonstrated in nuclei and mitochondria of rat brain cells following injury, its distribution within human atheroma as well as its precise cellular location still remains elusive.
Methods: By immunohistochemistry and immunoelectron microscopy the expression of PARP-1 and iNOS in surgical specimens from healthy and atherosclerotic human aorta was investigated.
Results: In the aortic plaques, our data demonstrated an upregulation of both iNOS and PARP-1 with respect to the healthy controls in which the expression of these enzymes was never investigated. Interestingly, immunoelectron microscopy showed that PARP-1 localizes exclusively in the nuclei of lesional smooth muscle cells and its expression strongly correlates with alterations in mitochondrial morphology.
Conclusions: Reactive oxygen and nitrogen species unbalance mitochondrial metabolism and under oxidative stress the activation of PARP-1 impairs mitochondrial functions. Our data suggest that the pro-oxidant environment of the plaque affects mitochondria morphology via a PARP-1 mediated mechanism since in the cells where the enzyme was absent no changes were observed in mitochondrial ultrastructure.The pharmacological modulation of iNOS and PARP-1 may represent a new strategy to prevent mitochondrial damage, exerting beneficial effects in the pathophysiology of the human atherosclerosis.

CDP 05. Effects of Xanthohumol, a Prenylated Chalcone Isolated from Humulus lupulus L., on Apoptosis of Human Glioblastoma Cell Lines.

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Background: Xanthohumol (XH), a prenylated chalcone of Humulus lupulus L., has shown chemopreventive and chemoterapeutic activities in several human malignancies (Zanoli & Zavatti,2008). TNF-related apoptosis-inducing ligand (TRAIL) is an attractive anticancer agent owing to its ability to trigger apoptosis selectively in cancer cells but not in normal cells (Jacquemin et al, 2010). The aim of this study was to investigate the effects of XH on apoptosis in human glioblastoma (GBM) T98G and U87MG cells.
Methods: Apoptosis was analyzed by flow cytometry. Intracellular ROS production was evaluated measuring DCF fluorescence by flow cytometry and fluorescence microscope. Mitochondrial membrane potential ($\Delta\Psi m$) was assessed by FACS analysis of TMRE positive cells. Western blotting analysis evaluated changes of MAP kinases phosphorylation and proteins expression.
Results: XH decreased the viability of T98G cells by induction of apoptosis in a time- and concentration-dependent manner whereas a small effect was observed at the highest concentration in U87MG cells. XH-induced apoptosis in T98G cells was associated with activation of caspases, PARP cleavage and mitochondria depolarization. ROS production appeared essential for the activation of the mitochondrial pathway and induction of apoptosis after XH exposure. Oxidative stress due to XH treatment is associated with ERK1/2 and p38 phosphorylation that was significantly abrogated using antioxidant agent NAC. MAPK-induced cell death after XH treatment was significantly reduced by specific ERK1/2 inhibitor PD98059 and p38 inhibitor SB203580. XH was also able to sensitize U87-MG cells to TRAIL-induced apoptosis.
Conclusions: These findings suggest that XH has a potential as a chemotherapeutic agent for GBM treatment.

CDP 06. Paradoxical Role of β-Catenin in Two Modes of Hepatocyte Death: Fas Versus LPS-Induced Liver Injury

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Background: β-catenin plays multiple roles in liver health and disease; however, its role in hepatic injury remains unexplored. Suppression of β-catenin signaling in hepatocytes causes a basal increase in apoptosis through unknown mechanisms. Here, we demonstrate that loss of β-catenin exacerbates Fas-mediated apoptosis but intriguingly, protects against TNF-α mediated apoptosis.
Methods: Wild-type (WT) and β-catenin conditional knockout (KO) mice were injected with either Jo-2 antibody (which activates Fas pathway) or galactosamine (GalN) followed by lipopolysaccharide (LPS) (which activates TNF-α pathway) and monitored for morbidity.
Results: Jo-2-injected KO mice show decreased survival as compared to WT, with extensive loss of liver architecture. KO livers show 9-fold increase in HGF expression, while Met protein levels are decreased. Additionally, Fas and β-catenin co-precipitate in unstimulated WT livers, suggesting a role for β-catenin in regulating the Fas-Met complex. Surprisingly, KO mice are refractory to GalN/LPS treatment: the mice show decreased morbidity compared to WT. Analysis of liver enzymes and TUNEL staining confirms the presence of massive injury and apoptosis in WT but not in KO mice. The anti-apoptotic transcription factor NF-κB is basally active in KO under unstimulated conditions, and NF-κB and its downstream targets are also elevated in KO mice after insult.
Conclusions: Our results demonstrate that deletion of β-catenin from hepatocytes confers protection from TNF-α induced apoptosis, perhaps through a compensatory upregulation of the NF-κB pathway, while the increased susceptibility of KO mice to Fas-mediated cell death might be due to decreased Met-Fas stability.

CDP 07. Involvement of Akt/NFκB Pathway in N6-Isopentenyladenosine-Induced Apoptosis in Human Breast Cancer Cells

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Background: N6-isopentenyladenosine (i6A) inhibits tumour cell growth by inducing cell apoptosis in various cancer cell lines such as colon cancer, sarcoma, and myeloma cells. In this study, we explored the molecular mechanisms of i6A as an anticancer agent on human breast cancer cell line MDA MB 231.
Methods: MDA MB 231 cell viability and death have been performed by MTT assay and 3H-thymidine incorporation. After cells had been cultured for 24 hr with different concentrations of i6A, proliferative response was estimated by colorimetric MTT-test. Inhibition of cell proliferation by i6A was measured by 3H-thymidine incorporation. Cell cycle progression has been assessed by flow cytometry and the expression of apoptotic markers and cyclins have been evaluated by western blot.
Results: Treatment with i6A decreased the cell proliferation of MDA MB 231 cells in a dose-dependent manner by arresting the cells at G0/G1 phase. This effect was strongly associated with concomitant decrease in the level of cyclin D1, cyclin E, cdk2, and increase of p21waf1 and p27kip. In addition i6A also induced apoptotic cell death by increasing the expression of Bax, and decreasing the levels of Bcl-2 and Bcl-xL, and subsequently triggered mitochondria apoptotic pathway. We observed that i6A suppressed the NFκB pathway and inhibited Akt activation.
Conclusions: The results of this study indicate that i6A decrease cell proliferation and induce apoptotic cell death in human breast cancer cells, possibly by decreasing signal transduction through the Akt/ NFκB cell survival pathway.

CDP 08. Effects of Autophagy Activation in Pancreatic Beta Cells

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Background: Beta cell loss mainly due to increased apoptosis contributes to development of type 2 diabetes (T2D). Recently, we observed a massive autophagic vacuole overload in beta-cells of diabetic patients, suggesting that non-apoptotic autophagy-associated cell death might also occur in T2D. Our aim was to investigate the effects of glucose and free fatty acids on autophagy activation in pancreatic beta-cells.
Methods: A well-differentiated beta-cell line (INS-1E) and isolated rat and human islets were incubated for various time periods at different concentrations of glucose and/or palmitic acid. Then, cell survival was evaluated and autophagy activation was investigated by a) monodansyl cadaverine (MDC) fluorescence; b) activation of LC3 protein c) ultrastructural observation and morphometric analysis of autophagic
Results: In INS-1E cells and rat and human islets, 0.5 or 1 mM palmitate markedly activated autophagy in beta-cells, whereas high glucose alone was ineffective. In particular, the proportion of cells with MDC-stained dots and the expression of the activated LC3-II band in western blots markedly increased after 6h of palmitate incubation. This was associated with unchanged beclin-1 and ATG1 gene expression, and decreased LAMP2 mRNA. These effects were maintained up to 18-24h incubation and were associated with a decline of cell survival correlated with both palmitate concentration and incubation time. Ultrastructural analysis showed that autophagy activation was associated with a remarkable swelling of the endoplasmic reticulum.
Conclusions: High free fatty acid concentrations might play a role in activation of autophagy in beta-cells, eventually leading to irreversible cell damage, through a mechanism that might involve endoplasmic reticulum

CDP 09. MicroRNA Involved In TRAIL Resistance In Human Glioblastoma Cells

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Background: Gliomas are the most frequent brain tumors and are still related to high morbidity and mortality. Despite progress in surgery and adiuvant therapy, glioblastoma patients still have a very poor prognosis because of incomplete resection and resistance to chemo- and radio-therapy.
Methods: To define novel pathways that regulate susceptibility to TRAIL in glioma, we have performed genome-wide expression profiling of microRNAs. Through MTT assay, Annexin V staining and Colony assay we have studied microRNAs-induced TRAIL resistance and by realtime-PCR and western blot we have investigated potential targets.
Results: We showed that TRAIL-resistant glioma cells (LN229 and TB10) expressed different levels of miRs if compared to TRAIL sensitive cells (LN18 and T98G). We focused our attention in particular on miR-21 and -30c. Their overexpression into T98G-sensitive cells induced TRAIL resistance, probably targeting interesting genes that could be related to TRAIL signaling pathway.
Conclusions: Accumulating evidence indicates that modulation of miR activity, regulating extensive gene expression networks, could open a new scenario in therapeutic strategies and represent an attractive strategy to achieve clinical benefits.

CDP 10. Pro-apoptotic Effect of Methylguanidine on Hydrogen Peroxide-treated Rat Glioma Cell Line

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Background: Brain tissue is particularly vulnerable to oxidative damage, possibly due to its high consumption of oxygen and many studies highlighted the role of excessive oxidative stress as inducer of apoptosis in numerous neurodegenerative disease. Neurological complications, such as dementia, are associated to the pathophysiology of the uremic syndrome and largely contribute to the morbidity and mortality in patients with renal failure. The uremic state is characterized by the retention of catabolytes, such as methylguanidine (MG) that possibly act as uremic neurotoxins. Here we report the effect of MG on oxidative stress-induced apoptosis in rat glioma cell line (C6) in vitro.
Methods: The oxidative stress was induced by hydrogen peroxide (H_2O_2; 1 mmol/L) and uremic conditions were simulated by pre-incubation of C6 cells with MG (0.1-10mmol/L) 1h before H_2O_2.
Results: MG alone didn't affect cell viability, but it significantly increased cell death induced by H_2O_2, as assessed by MTT assay. This effect could be relate to the enhance in H_2O_2 pro-apoptotic effect on C6 cells. The fluorescent dye FURA 2-AM test on H_2O_2-treated C6 cells showed that MG induced significant raise in [Ca2+]i, mainly for depolarizing mitochondrial membrane potential, as indicated by p-trifluoromethoxy-pyhenylhydrazone. Furthermore, MG in a concentration-dependent manner, significantly increased H_2O_2-induced Bax expression, activation of caspase-3 and PARP in C6 cells.
Conclusions: This study firstly reports that MG potentiated the pro-apoptotic effect of oxidative stress in rat glial cells, showing the involvement of factors regulating the apoptotic pathway and mostly mithocondrial calcium homeostasis.

CDP 11. Role of Calpains in Melanoma Cells

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Background: In the last few years, we focused on the involvement of calpains, Ca2+-dependent cysteine proteases, in melanoma cell biology. In cisplatin-treated melanoma cells we have previously demonstrated that conventional calpains (1 and 2) are early activated, and their pharmacological inhibition protects from apoptosis, through down-regulation of cisplatin-induced p53 activation. More recently, in melanoma cell lines and melanocytic lesions we have identified and sequenced two novel splicing variants of muscle-specific calpain 3. In cisplatin-treated cells, an increased transcription and (auto)proteolytic activation of these variants occurs. These events, likely through a cross-talking with conventional calpains, are correlated to the apoptotic response. Consistently, the expression of these variants is significantly lower, compared to benign nevi and to early stage melanocytic lesions, in vertical growth phase melanomas and metastases, the most aggressive lesions, usually resistant to apoptosis.
Results: Preliminary results show that inhibition of conventional calpains also elicits an autophagic response, suggesting that active calpains are somehow able to interfere, in a mTOR-independent pathway, with a pro-survival autophagic cascade. Interestingly, trehalose-induced autophagic response, while preventing apoptosis, is also able to prevent calpain activation, suggesting that autophagy can exert its pro-survival effect by down-regulating conventional calpains. Preliminary results obtained in transient transfections of the longer calpain 3 variant show that highly expressing cells soon detach and die; transfected cells showing a lower protein level (full-length and proteolytic fragment) survive but show a remarkable increase of cisplatin-induced apoptosis.
Conclusions: Conventional calpains (likely through inhibition of autophagy) and calpain 3 variants exert a pro-apoptotic role in melanoma cells.

CDP 12. Epigenetic Changes, DNA Oxidation and Formation of Chromatin Loops During Transcription Induced by Retinoic Acid

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Background: Eukaryotic transcription shows an intrinsic periodicity, that determines the cyclical nature of genic expression. Retinoic Acid binds its cognate receptor and assembles the transcription initiation complex on specific DNA sites (RARE). The transcriptional regulation involves several mechanisms such as the positioning of nucleosomes, chromatin-remodelling and histone post-translational modifications.
Methods: To analyze the loop formation, the overall spatial organization of chromosomes and their physical properties at high resolution we used the 3C technique using MCF7 cells for all experiments. The products were analyzed by PCR. To confirm our data we used ChIP analysis using specific antibodies.To study histones modifications before ChIP we transfected the cells with specific siRNA and a mutant demethylase.
Results: Upon activation of RA receptor, on the RARE-promoter chromatin of caspase 9 gene, histone H3 lysine 9 undergoes demethylation, catalyzed by lysine specific demethylase, LSD1 and JMJD2A generating an oxidation wave, that modifies the DNA locally and recruits OGG1 and APE1, involved in base excision repair.These events are essential for the formation of chromatin loop(s) juxtaposing the RARE with the promoter region and the poly-A region. The Promoter-RARE interaction is also detectable in the absence of RA. The loop between the promoter and Poly-A is induced by stimulation. Finally, the interaction between RARE and poly-A is cyclical.
Conclusions: The RNA PolII contacts both the active receptor lying on the RARE and the SSU72 protein at the polyadenylation site. The receptor bound on the RARE governs the 5' and 3' end selection, directing the productive transcription cycle of RNA polymerase.

CELL SIGNALING AND ADHESION

CSA 01. Characterization of F2-isoprostane Signaling in Hepatic Stellate Cells

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Background: Hepatic stellate cells (HSC) are implicated in the pathogenesis of liver fibrosis, where oxidative stress is thought to play a key role. We previously demonstrated that F2-isoprostanes, generated during CCl_4-induced hepatic fibrosis, mediate HSC proliferation and collagen hyperproduction and these effects are mediated by thromboxane receptors (TP). In this study, we examined which signal transduction pathways are set into motion by isoprostanes to exert their fibrogenic effects.
Methods: HSC were isolated from the rat liver and treated with 8-epi-PGE2α. Ins(1,4,5)P3 (IP3) and cAMP levels were determined by commercial kits. Activation of mitogen-activated protein kinases (MAPK) and cyclin 1 was assessed by Western blotting. Cell proliferation and collagen production were assessed by measuring the incorporation of 3H-thymidine and 3H proline, respectively.
Results: 8-epi-PGE2α increased 4 times IP3 and affected cAMP production. The expression of cyclin D1, a molecule involved in cell proliferation, is also increased. Furthermore, 8-epi-PGE2α activated two classes of MAPK: extracellular signal-regulated

kinase (ERK) and p38, which have been shown to influence cell proliferation and collagen gene expression.
Conclusions: On the basis of these data, it is possible to hypothesize that one of the pathways activated by 8-epi-PGF2α is that of Gq/PKC. The binding of isoprostane to TP could stimulate downstream MAPK activation, via PKC. In particular, p38 is known to increase in HSC collagen production, while ERK is able to increase cyclin D1 expression and then cell proliferation. Thus, fibrogenic effects of isoprostanes in HSC are mediated through TP binding by specific activation of these transduction pathways.

CSA 02. Exposure of Healthy Resting Young Men to Normoxic Hypoxia Induces Rapid Reversible Reduction of Circulating Endothelial Progenitor Cells
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Background: Circulating endothelial progenitor cells (EPCs) are thought to contribute to the maintenance of endothelial function and tissue perfusion through mechanisms of endothelial repair and angiogenesis. EPCs are present at very low numbers in the adult peripheral blood, where they are identified based on the lack of leukocyte markers and the coexpression of stem cell markers and the VEGF-receptor KDR. Hypoxia can regulate the frequency of circulating EPCs by affecting their mobilization and tissue localization. While localized tissue ischemia induces an increase of circulating EPCs, the effects of systemic exposure to hypoxia have still to be clarified.
Methods: In this study we analyzed circulating EPCs in 9 young healthy men that were exposed for 4 h to continuous normobaric hypoxia, at conditions equivalent to an altitude of 4100 m. Peripheral venous blood samples were serially obtained before and at multiple time points during and after hypoxia exposure. To avoid the confounding effects of physical exercise, all the subjects were kept at rest during the study. EPCs were analyzed by 5-color flow cytometry directly performed on whole blood samples.
Results: A time-dependent significant decrease of EPCs was observed in all the subjects. EPCs decreased progressively during the first 2 h of hypoxia exposure, and maintained a plateau thereafter. A recovery to initial EPC values was observed in all but one subject within 4 h after hypoxia discontinuation. The mechanisms underlying these results are currently under investigation.
Conclusions: Our results provide novel insights into the comprehension of the effects of hypoxia on EPC biology.

CSA 03. miR221 and miR222 Regulate Cell Motility in Glioma Cell Lines Targeting the Protein Phosphates PTPmu
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Background: Glioblastoma multiforme (GBM) is the most frequent brain tumor in adults and is the most lethal form of human cancer. Despite the improvements in treatments, patient survival remains poor. Different evidence indicates that microRNAs might play a fundamental role in tumorigenesis, cell proliferation, migration and apoptosis.
Methods: Through a microarray analysis we identified different microRNAs upregulated in the tumorigenic glioma cell lines U87 compared with the non tumorigenic cell lines T98G.
Results: Among different microRNA we focused our attention on miR221 and miR222. By bioinformatic analysis we identified a binding site for these two miRs in the 3'UTR of the protein phosphatase PTPmu. Previous studies indicated that PTPmu suppresses cell migration and is downregulated in glioma. Significantly, we found that miR 221 and miR222 overexpression induced a downregulation of PTPmu as analyzed by both western blot and RT-PCR. Furthermore, miR222 and miR221 induced an increase in cell migration in glioma cells. Accordingly we found an inverse correlation between miR221and miR222 and PTPmu in glioma cancer samples, as analyzed by immunohistochemistry and in situ hybridization on a tissue array representing 46 glioma and normal brain tissues.
Conclusions: In conclusion, our results suggest that microRNA 221 and 222 might regualte glioma tumorigenesis through cell migration process control.

CSA 04. Chenodeoxycholic Acid (CDCA) through TGR5-Dependent CREB-Signaling Activation Enhances Cyclin D1 Expression and Promotes Human Endometrial Cancer Cell Proliferation.
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Background: Bile acids (BAs) are amphipathic detergents that are synthesized in the liver and stored in the gallbladder. In addition to their well-established role in dietary lipid absorption and cholesterol homeostasis, it has recently emerged that BAs can also act as signaling molecules, with systemic endocrine functions particularly in obese women in which endometrial cancer exhibits a strong incidence. This brought us to investigate the biological effects of different concentrations of the CDCA on human endometrial cancer Ishikawa cells.
Methods: Western blotting, RT-PCR, RNA interference, ChIP and EMSA assays; mutagenesis and gene reporter analysis.
Results: Low concentrations of CDCA were able to stimulate Ishikawa cell growth as evidenced by [3H]Thymidine incorporation and cell cycle analysis by inducing a significant increase in Cyclin D1(CD1) protein and mRNA expression. In contrast, high doses of CDCA showed cytotoxic effects with an increase of CDK inhibitor p21WAF1/CIP1 expression through a p53-indipendent pathway leading to apoptotic event as evidenced by PARP cleavage expression and DNA laddering assay. Moreover, mutagenesis studies, EMSA and ChIP analysis revealed that the cyclic AMP-responsive element (CRE) binding protein motif within CD1 proximal promoter was required for CDCA-induced CD1 expression. Silencing of a cell surface bile acid sensor TGR5 and CREB gene expression reversed the up-regulatory effect of low doses of CDCA on CD1 expression and Ishikawa cells proliferation.
Conclusions: Although many issues remain to be elucidated, BA and its membrane receptor could be implemented as a target in the novel strategies for endometrial cancer treatment.

CSA 05. Cellular Localization and Cytoskeletric Effects of Eight Different Arg Isoforms Transfected in COS-7 Cells
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Background: The non-receptor tyrosine kinase Arg (Abl related gene) is an essential regulator of cell migration and morphogenesis by attenuating actomyosin contractility and stimulating actin polymerization and cellular protrusions. Arg induces cytoskeletal rearrangements through its kinase and C-terminal domains containing two F-actin and one microtubule binding regions. Alternative splicing of 5'- and 3'- regions of the human Arg transcript gives rise to eight isoforms(1BLCTL, 1BSCTL, 1BLCTS, 1BSCTS, 1ALCTL, 1ASCTL, 1ALCTS, 1ASCTS), with differences at N- termini and at first C-terminal F-actin binding domain. These eight isoforms are differently expressed in normal and neoplastic cells.
Methods: We analysed with immunofluorescence analysis by confocal microscopy and western blot the cellular localization and functional role of the eight different Arg isoforms, cloned into the pFlag CMV-2 expression vector and transfected in COS-7 cells, in regulating cell morphology, adhesion and cytoskeletal organization.
Results: All Arg isoforms show similar cytoplasmatic distribution, except for 1BSCTS that has a specific nuclear localization confirmed also in other cell types. ImageJ software analysis of immunofluorence pictures evidenced that the overexpression of the eight Arg isoforms differently inhibits actin stress fiber formation and cell spreading and variably induces filopodia-like cytoplasmatic protrusions. Studies to prove the involvement of the different Arg isoforms in Rho-family GTPase pathways, probably responsible of these cellular morphological effects, have been also performed.
Conclusions: The different expression of Arg isoforms previously described in normal and neoplastic cells, and their different role in the modulation of cell morphology and cytoskeleton organization might be important for metastatic diffusion.

CSA 06. Computational and Experimental Characterization of Two Nuclear Localization Signals and a Nuclear Export Signal that Modulate BCR Intracellular Localization
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Background: The Breakpoint Cluster Region (BCR) gene contributes to the pathogenesis of Chronic Myeloid Leukemia (CML) by fusing its first exons to most of the ABL gene sequence. We decided to investigate the mechanisms regulating the intracellular localization of BCR and to determine if they applied to the BCR-ABL chimaeric oncoprotein of CML.
Methods: In the BCR sequence, we computationally defined two putative Nuclear Localization Signals (NLS) and one Nuclear Export Sequence (NES). We then generated both NLS and NES GFP-fusion constructs and evaluated their intracellular localization by immunofluorescence. We also generated both BCR and BCR-ABL mutants devoid of each NLS and NES and transiently expressed them in HeLa or BaF3 cells to investigate their expression, localization and transforming ability.
Results: Both NLS sequences directed GFP in the nucleus, while the NES favored its cytoplasmic localization. However, wild-type BCR was confined to the cytoplasm. Removal of the oligomerization domain (OD), of the Protein Kinase C region 2 (DC2) and of the Rho-GAP domain induced BCR nuclear staining. Since only the OD and part of the DC2 are preserved in the CML oncoprotein, we assembled a ΔDO-ΔDC2 BCR-ABL. This construct remained in the cytoplasm but failed to transform BaF3 cells, leading instead to their killing.
Conclusions: Our results suggest that BCR displays two putative NLSs and one NES that are inhibited by the OD, DC2 and Rho-GAP domains. A kinase-active BCR-ABL lacking these regions retains its cytoplasmic localization but is devoid of transforming activity and induces massive cell death.

CSA 07. Akt2 Inhibition Enables the Forkhead Transcription Factor FoxO3a to a Repressive Role for ERα Transcriptional Activity in Breast Cancer Cells

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Background: Estrogen receptor α (ER) and the insulin-like growth factor I receptor (IGF-IR) pathways are engaged in a functional cross-talk in breast cancer, promoting tumor progression and increased resistance to anticancer treatments and radiotherapy. Here we introduce new mechanisms through which proteins of the IGF-I/IGF-IR signaling pathway may regulate ER function in the absence of ligand.
Methods: MCF-7 breast cancer cells were used. To assess cell specificity, all the experiments were conducted in two ER-negative cell lines, HeLa and SKBR3, ectopically expressing ER. The methods used were: Western blotting and immunoprecipitation; Chromatin immunoprecipitation; gene reporter and expression; and confocal microscopy.
Results: Our results indicate that in ER-positive breast cancer cells, Akt2 modulates ER transcriptional activity at multiple levels, including 1) the regulation of ER expression and its nuclear retention and 2) the activation of one of its downstream targets, the forkhead transcription factor FoxO3a. FoxO3a co-localizes and co-precipitates with ER in the nucleus, where it binds to forkhead responsive sequences on ER target pS2/TFF-1 promoter; in addition, FoxO3a silencing leads to an increase of ER transcriptional activity, suggesting a repressive role of the forkhead transcription factor on ER function. Moreover, 17β-estradiol up-regulates FoxO3a levels, which could represent the basis for an ER mediated homeostatic mechanism.
Conclusions: These findings provide further evidence of the importance of proteins of the growth factors signaling in ER regulation, introducing Akt2/FoxO3a axis as a pursuable target in ER-positive breast cancer therapy.

CSA 08. Investigation of RHO GTPase Involvement in Urokinase-Dependent Migration and Invasion.

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Background: The urokinase-type plasminogen activator (uPA) and its cognate receptor (uPAR) play a central role in cell adhesion and migration. In human malignant tumours, the overexpression of uPA and uPAR are associated with a high risk of metastases. A more detailed knowledge of cell-signaling pathways regulating cell migration and invasion and activated by the uPA-uPAR axis could therefore lead to the development of selective inhibitors. uPA has been implicated in human prostate cancer.
Methods: PC3 cells were transfected with a siRNAs designed to silence most of the 20 human Rho GTPases. Transfected cells were tested for their ability to migrate in 2D, in response to uPAR engagement with the Amino Terminal Fragment of uPA (ATF). The analysis was carried out in Transwells and by scratch wound-healing. In addition, the ability of transfected cells to invade Matrigel upon uPA-stimulation was tested.
Results: The results showed that the silencing of RhoB, RhoC and RhoF reduced ATF-dependent migration. In order to identify downstream molecules involved in ATF-dependent cell motility, we analyzed the activation of paxillin and cofilin, which are involved in cytoskeletal rearrangements. We found that ATF led to an increase in paxillin and cofilin phosphorylation in control PC3 cells. The silencing of RhoB, RhoC and RhoF decreased ATF-induced paxillin and cofilin activation.
Conclusions: These data suggest that the uPA/uPAR system promotes migration in prostate cancer cells through RhoB, RhoC and RhoF, but not through RhoA. The inhibition of the mediators of uPA/uPAR system could represent a novel strategy to interfere with pathologic processes involving cell migration.

CSA 09. HINT1 Can Act as a Tumor Suppressor Gene in Melanoma Cells by Negatively Modulating Key Oncogenic Pathways.

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Background: HINT1 is a haplo-insufficient tumor suppressor gene involved in several cell pathways important for human tumorigenesis. HINT1 inhibits the activity of key transcription factors like β-catenin and AP-1 in epithelial cells and MITF in mast cells. MITF is a transcription factor whose inactivating mutations produce loss of viable melanocytes and whose gene is amplified in a subset of human melanomas.
Methods: The aim of this study was to investigate the potential role of HINT1 in melanoma genesis.
Results: SK-Mel-28 human melanoma cells express low levels of HINT1 protein. We found that constitutive expression of the HINT1 full length cDNA significantly impairs SK-Mel-28 proliferation in vitro and tumorigenesis in vivo. These effects were associated with a decreased expression of cyclin D1 and Bcl-2, well known MITF transcription targets.Using luciferase reporter assays, we found that HINT1 inhibits the activity of luciferase reporters under the Bcl-2 and cyclin D1 promoters. We also demonstrated that HINT1 directly interact with MITF and β-catenin and binds the chromatin at the MITF and β-catenin sites in the Bcl-2 and cyclin D1 promoter regions.Transcription factor activity is often modulated by the interaction with regulatory proteins and we found that HDAC1 and mSIN3A can be co-immunoprecipitated with an anti-HINT1 antibody. We also detected an increase of the binding of HDAC1 to the E-BOX sites in Bcl-2 promoter upon HINT1 overexpression.
Conclusions: Overall these data suggest that HINT1 can promote a transcriptional repressive state at target gene promoters and modulate the expression of well-characterized transcriptional targets in melanoma cells.

CSA 10. Role of p85α PI3K Mutants on the Insulin Regulation of Estrogen Receptor-Positive MCF-7 Cell Growth and Motility.

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Background: Insulin and IGF are signaling molecules essential for cell survival, transformation and hormone-independent growth of MCF-7 cells. IRS-1 and IRS-2 docking protein are the major downstream signaling proteins for insulin and IGF1 receptors, conveying signals to the PI-3K/Akt pathway. The PKA *in vivo* phosphorylates Ser83 adjacent to N-terminus SH3 domain in the PI3K regulatory subunit, p85α and influences the ability of SH3 domain to interact with different partners like as estrogen receptor and RIIβ. The aim of study was to evaluate the effect of p85α mutants on MCF-7 growth and motility regulation.
Methods: MCF-7 cells expressing p85α WT or Ser83 mutants were stimulated with Insulin or IGF-1. Total proteins were immunoprecipitated with p85α antibody and analyzed with Western blot; Cell cycle and migration were analyzed with FACS and Wound Healing assay.
Results: Here we demonstrate that MCF-7 cells expressing the Ser83-> Asp mutant of p85α (p85D) show a higher rate of G1 arrest with respect to the Ser83-> Ala mutant (p85A); furthermore mutant expression modified the cell response to insulin treatment. The p85A mutant displayed higher proliferation rate and a significant reduction of motility, compared to the wild-type and to the aspartic acid mutant, suggesting an involvement of p85 Ser 83 in the regulation of cell motility.
Conclusions: We speculate that the Ser 83 is crucial for mitogen signal transduction. Therefore identification of mutations in the SH3 domain region that could influence protein-protein interactions, might be informative for prognosis and predictive for adequate therapies in breast cancer.

CSA 11. The Role of Androgen Receptor/Filamin A Association in Androgen-Induced Cell Migration.

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Background: In different cell types androgens rapidly trigger signalling pathway activity well-known to be regulated by growth factors, cytokines and extracellular matrix. This so-called 'non-genomic' action occurs within seconds or minutes and is independent of steroid receptor transcriptional activity. Androgen signalling has been correlated to cell migration in different cell types. However, the molecular mechanism underlying this process is poorly understood.
Methods: We used NIH3T3 fibroblasts, since these cells harbour a classical AR devoid of transcriptional activity. Nevertheless, this receptor rapidly activates signalling effectors upon stimulation with physiological androgen concentration (10 nM; Castoria et al., 2003).
Results: By combining different approaches, we observed that filamin A plays a regulatory role in androgen-regulated migration. Ten nM R1881 induces association and co-localization of AR with filamin A (FlnA) at cytoskeleton of NIH3T3 fibroblasts. This complex also recruits integrin beta 1. Assembly of AR/FlnA/integrin β1 complex by androgens induces activation of Rac 1 and FAK, which both coordinate migration.
Conclusions: Our findings show a novel and unexpected role for interaction AR/FlnA in extra-nuclear compartment of target cells. Such an interaction may impact human organ development as well as cancer progression and may offer a new target to gain a more tailored therapy of androgen-dependent human cancers.

CSA 12. EDF-1 Participates to the Regulation of Nitric Oxide Release in VEGF Treated Human Endothelial Cells

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Background: Vascular endothelial growth factor (VEGF) induces nitric oxide (NO) release by triggering multiple intracellular signals, among which the calcium/calmodulin pathway and the activation of Akt, events which induce endothelial NO synthase (eNOS) activity. Because Endothelial Differentiation-related Factor (EDF)-1 is a calmodulin binding protein and plays a role in modulating endothelial functions, we evaluated whether EDF-1 is implicated in the regulation of eNOS activity in VEGF-treated human endothelial cells.

Methods: Human umbilical vein endothelial cells (HUVEC) were utilized. EDF-1 was silenced by shRNA. Protein-protein interactions were determined by co-immunoprecipitation. Nitric oxide release was measured by the Griess method.
Results: VEGF promotes the dissociation of calmodulin from EDF-1, an event which correlates with the increase of calmodulin bound to eNOS and the induction of NO release. Endothelial cells silencing EDF-1 produce more NO than controls and do not increase NO release in response to VEGF. The insensitivity to VEGF results from the incapability of cells silencing EDF-1 to phosphorylate eNOS Ser1177. Interestingly, okadaic acid, a pharmacologic inhibitor of the serine/threonine phosphatase PP2A, which preferentially dephosphorylates eNOS Ser1177, restores NO release and eNOS Ser1177 phosphorylation in cells silencing EDF-1.
Conclusions: Our results suggest EDF-1 as a novel contributor to the complex regulation of eNOS activity in human endothelial cells.

CSA 13. Phosphoproteomic Analysis of Differentially Ligated Human Neutrophils Elucidates Downstream Tyrosine Phosphorylation Events
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Background: A number of neutrophil functions have been shown to be altered, including adhesion, diapedesis and chemotaxis, depending on differential ligation of cell surface receptors such as integrins. The β-glucans are a class of long-chain polymers of glucose that have anti-infective and immune modulatory effects, including the ability to enhance phagocytosis and microbial killing. Cellular signal transduction comprises modifications such as reversible phosphorylation by which cells convert an extracellular stimulus to an intracellular response (outside-inside). We hypothesize that β-glucan supplementation of fibronectin changes tyrosine phosphorylation signaling leading to different cellular behaviors.
Methods: Tandem mass spectrometry was used to provide peptide identification and quantification by performing a survey scan in the FT-ICR followed by data-dependent MS/MS scans of the nine most abundant ions in the LTQ.
Results: Using this approach, we found significant changes in a number of sites of tyrosine phosphorylation in neutrophils exposed to immobilized fibronectin +/- IMPRIME-PGG-β-glucan. One such site is ERK1 (Y204), which is upregulated in neutrophils that have been treated with fibronectin + β-glucan in contrast to those that have been treated with fibronectin. This phosphopeptide has been validated via western blot and biological methodologies.
Conclusions: Using elegant methods such as described here provides a global analysis of alterations in phosphorylation in using a correlative approach that has the capability of providing enough information to determine accurate predictions of how specific phosphorylation modifications can be interrelated within pathways. Ultimately, these studies are expected to render new pathways for neutrophil signaling, providing fundamental targets for pharmacological therapies.

CSA 14. Sgk1: A Novel Master-regulator of Nuclear Export and Genomic Stability in Neoplastic Epithelial Models.
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Background: Sgk1 is a kinase that is activated by insulin, Igf1 and Interleukin 2 through PDK1/PDK2 . PDK1 binds phospho-S422 in the hydrophobic motif (H-motif) of SGK1 to phosphorylate T256. The Hmotif kinase that phosphorylates Sgk1 at S422 to prime it for phosphorylation by PDK1 has been recently identified as mTOR. The Sgk1 dependent p27 phosphorylation determines G1 progression.Sgk1 also transduces insulin, growth factor and steroids dependent survival signals. We recently demonstrated that Insulin activated Sgk1 down-regulates p53 through MDM2 that directs p53 to ubiquitylation and proteosomal degradation. Preliminary data show that the activity of Sgk1 is also necessary to export p53 from the nucleus to the cytoplasm where it is inactivated by ubiquitylation.
Methods: By 2DGE and mass spectrometry we found that the silencing for Sgk1 or its overexpression by a specific adenovirus infection in colon cancer cell affects the expression of proteins such as RanGTP, RANBP1 and CRM1 involved in the nuclear export and in genomic stability.
Results: Moreover, our experiments demonstrate that sgk1 is a strong transcriptional regulator of RanBP1. RanBP1 activity is required for the organization and function of the mitotic spindle. Infact Sgk1 specific silencing was associated with a reduced number of aneuploid metaphases in Hela and RKO cells.
Conclusions: Sgk1 interacting peptides, identified by phage display, can be used to design small molecules that may find an application in the treatment of human tumors.

CSA 15. Regulation of Intestinal Epithelial Barrier by Desmosomal Cadherins
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Background: The gastrointestinal epithelium acts as a highly selective barrier that interfaces distinct luminal and tissue environments. The importance of intact intercellular contacts is highlighted by pathological disorders of the gut such as inflammatory bowel disease, in which compromised epithelial barrier leads to inflammation and ultimately cancer. Intercellular junctions that encompass the Apical Junctional Complex and Desmosomes are comprised of transmembrane, membrane-associated and signaling proteins that are organized in distinct spatially restricted complexes. In addition to serving as gatekeepers of the epithelium, junctional complexes relay extracellular stimuli and coordinate epithelial proliferation, differentiation and apoptosis.
Methods: Complementary *in vitro* cell culture and *in vivo* mouse models were utilized for these studies.
Results: An active role of Desmosomal Cadherins in regulating intestinal epithelial cell proliferation, apoptosis and ultimately ultimately barrier function was observed. Additionally, we have identified the signaling pathways by which these proteins control epithelial biological responses.
Conclusions: These studies emphasize the critical pluripotent function of epithelial intercellular junction proteins and highlight their vital contribution in regulating intestinal epithelial barrier function.

HOST-PATHOGEN INTERACTIONS

HP 01. Reduction of Volatile Sulphur Compounds Production in *H. Pylori* Positive Patients Suffering from Halitosis after Successful Eradicating Therapy.
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Background: Halitosis is a general term used to describe an unpleasant odour emanating from the oral cavity. Previous reports of volatile sulfur compounds (VSCs) in *Helicobacter pylori* (Hp) cultures and the amelioration of halitosis after eradication suggested a causal link between Hp infection and halitosis. Aim of this study was to investigate the outcome of halitosis before and after eradicating therapy in Hp+ patients with functional dyspepsia (FD).
Methods: All patients (3M and 7F, median age 51,5 range 33-66 yrs) were Hp+ and self complaining of halitosis. Before administering eradicating therapy (pantoprazole + claritrhomycin + amoxicillin), patients were subjected to a GSRS questionnaire for dyspeptic symptoms, organoleptic (OLS) assessment of halitosis (5 points scale from 0=absent to 5= extremely foul odour) and Halimeter test for measuring VSCs. The same procedures were followed 8 weeks after the end of eradicating therapy.
Results: Hp infection was eradicated in 8/10 patients. Eradication resulted in significant relief of dyspeptic symptoms [GSRS score 49 (25-68) vs. 33 (22-48), p=0,008], OLS score [3 (2-4) vs. 1,5 (0-3) p=0,03] and VSC score [239 (118-1586) vs. 165 (15-621) p=0,03] (Wilcoxon Signed Rank Test).
Conclusions: Eradication of *H. pylori* in patients with functional dyspepsia and halitosis resulted in resolution of halitosis and overall reduction in dyspeptic symptoms. Of note, also the production of VSCs was significantly reduced by antibiotic treatment. This finding supports the existence of a link between Hp infection and halitosis and suggests that Hp eradication may represent a therapeutical option in patients with halitosis.

HP 02. *Helicobacter Pylori* L-asparaginase: from Gene to Diagnostic Tool
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Background: *Helicobacter pylori* is one of the most common human pathogens classified by WHO as a class I carcinogen. In our previous work, we have noticed that the bacterial broth culture filtrate (BCF) can interfere with the proliferation of cultured cells. We here describe the discovery of a novel bacterial factor able to inhibit the cell-cycle of exposed cells.
Methods: An integrated approach was adopted including: optimisation of bacterial culture conditions, size exclusion chromatography, non-reducing gel electrophoresis, mass spectrometry and enzymatic assays.
Results: L-asparaginase was identified as the factor responsible for the cell-cycle inhibition. Its effect was confirmed by suicidal inhibitors, a knock-out strain and the action of the recombinant protein on several cell lines. L-asparaginase could also stimulate the immune response of a subset of infected patients, otherwise classified as negative.These results confirm that *H. pylori* L-asparaginase is the main actor in the cell-cycle inhibition of cells exposed to BCF and it could act *in vitro* as a pathogenic factor. On the other hand, preliminary data collected by our group show that the gastric epithelium displays a differential expression of the concurrent enzyme asparagine synthetase, thus suggesting that a potential differential interference by L-asparaginase in the proliferation of the cells exposed to the bacterium might also be a relevant pathogenic mechanism *in vivo*.
Conclusions: L-asparaginase is a pathogenetic factor which could have diagnostic applications and, as other enzymes of the same class, also therapeutic applications.

HP 03. LPS-Induced Chromatin and DNA Methylation Changes in Human Intestinal Epithelial Cells

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Background: A possible novel additional strategy used by bacterial pathogens during infection is to interfere with host cellular processes by inducing epigenetic modifications and consequently determining a new specific cell transcriptional profile. These mechanisms may operate at a gene-specific level and include chromatin modifications, orchestrated by chromatin-remodeling complexes and histone-modifying enzymes, and DNA methylation directed by DNA-methyl transferases. The release of LPS by bacteria is mainly responsible for systemic reactions and stimulates both immune and specific epithelial cell types to release inflammatory mediators. It is known that LPS induces the release of IL-8 by intestinal mucosal cells. Because bacteria may induce alteration of epigenetic patterns in host cells, we have investigated whether LPS induced IL-8 activation in human intestinal epithelial cells involves changes of histone modifications and/or DNA methylation at the IL-8 gene regulatory region.
Methods: Chromatin-immunoprecipitation (ChIP), DNA methylation analysis by MALDI-TOF.
Results: Our data demonstrate an important role of histone modifications, including histone H3 acetylation and H3K4, H3K9 and H3K27 methylation state, in LPS-mediated IL-8 gene activation in intestinal epithelial cells. In particular we demonstrate that H3-acetyl, H3K4me2 and H3K9me2 changes are early, transient and correlate with the modulation of transcriptional activity. Conversely, increase of H3K27me3 levels at IL-8 gene are later and long lasting.
Conclusions: Our data raise the intriguing possibility that the observed chromatin modifications could be potentially involved in two important phenomena such as the LPS tolerance, and the hypermethylation of PcG target genes in intestinal cancer.

HP 04. Mechanisms Utilized by *Francisella Tularensis* to Invade Hepatocytes

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Background: *Francisella tularensis* is a facultative intracellular bacterium that is the etiological agent of tularemia. The liver is an important target of infection in tularemia, and *F. tularensis* grows to high numbers within hepatocytes, the major cell type of this organ. This study investigates the mechanisms utilized by *F. tularensis* to invade hepatocytes.
Methods: Mechanisms of uptake of *F. tularensis* Live Vaccine Strain (LVS) were investigated in vitro using the murine AML-12 hepatocyte cell line. Additionally, an *F. tularensis* ssp. novicida transposon library was screened for mutants with altered uptake by hepatocytes.
Results: Addition of cytochalasin D to the hepatocytes blocked uptake of the LVS nearly completely. In contrast, bacteria killed by heating or formalin fixation were readily ingested by the hepatocytes. Treatment of the bacteria with chloramphenicol also had no effect on invasion. LVS mutants lacking components of pili or the type I secretion system retained their ability to be taken up by the hepatocytes. Screening of the *F. tularensis* ssp. novicida transposon library led to identification of two unclassified outer-membrane proteins, the absence of which enhanced uptake by both AML-12 cells and HH4 human hepatocytes.
Conclusions: These results show that polymerization of the actin cytoskeleton is needed for uptake of *F. tularensis* by hepatocytes. Additionally, there is no requirement for viability of or protein synthesis by the bacteria for this process. The mutants identified by the screen suggest that certain bacterial genes may negatively regulate the uptake of *F. tularensis* by hepatocytes. Further characterization of these genes is in progress.

HP 05. Flying Under the Radar: How *Francisella Tularensis* Evades Innate Immunity

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Background: *Francisella tularensis*, the bacterial agent of tularemia, has been categorized as a potential bioweapon due to its high infectivity and virulence. The exceptional virulence of *F. tularensis* probably stems from the organism's ability to avoid the immune defenses of its mammalian hosts. A key player in innate immunity is the vascular endothelium, which when activated recruits circulating leukocytes to areas of infection.
Methods: Cultured human umbilical vein endothelial cells (HUVEC) were incubated with *F. tularensis*, and activation of the endothelial cells was assessed by measuring their production of pro-inflammatory chemokines.
Results: Although killed *F. tularensis* activated HUVEC, the living bacteria did not. Moreover, living *F. tularensis* suppressed stimulation of the endothelial cells by the killed organisms. Suppression was dose- and contact-dependent and occurred rapidly. The endothelial protein C receptor (EPCR) confers anti-inflammatory properties when bound by activated protein C. When the EPCR was blocked, *F. tularensis* lost its ability to dampen stimulation of HUVEC.
Conclusions: This study provides the first demonstration that a pathogen can use the EPCR to diminish the protective response of endothelial cells. Given the critical role of endothelial cells in inflammation, suppression of their activation is likely a mechanism that *F. tularensis* uses to foster its own survival and dissemination.

HOST-PATHOGEN INTERACTIONS IN CANCER

HPC 01. Human Late-Endothelial Progenitor Cells as Putative Precursors of Kaposi Sarcoma Spindle Cells: They Support Persistent *In Vitro* Infection With Human Herpesvirus-8 and Acquire Virus-Induced Morphologic and Functional Features Similar to Spindle Cells

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Background: The predominant cells type in Kaposi's sarcoma (KS) lesions are the spindle cells that are of endothelial origin and are infected by HHV-8, the causative agent of KS. HHV-8 induces profound changes in multiple types of infected cells. We previously demonstrated that endothelial progenitor cells (EPCs) cultured *ex-vivo* as late-EPCs from KS patients are HHV-8-infected but do not differ in morphology and functions from control EPCs. Here we further investigated the effects of HHV-8 on late-EPCs.
Methods: Control late-EPCs were infected in vitro with HHV-8. HHV-8 genomes were quantified by real-time PCR analysis of cells and supernatants. The lytic and latent cycle phases of infection were assessed by rKSHV.219. The effects of HHV-8 infection on EPCs were evaluated in terms of cell morphology, viability, proliferation, immunophenotype and angiogenesis.
Results: Monitoring of cell infection in long-term cultures revealed the persistence of HHV-8 DNA in all the infected late-EPC cultures (up to 37 days). HHV-8 genomes were detected at variable levels during cultures, suggesting that lytic infection may spontaneously occur. This was confirmed by expression of lytic and latent genes in rKSHV.219-infected cells. HHV-8 infection induced late-EPCs to acquire a spindle shape and an activated immunophenotype, to upregulate CD49c and avb3 that are involved in HHV8 entry into the cells, to promote cell proliferation and stimulate angiogenic activity.
Conclusions: We conclude that, upon HHV-8 *in vitro* infection, EPCs support persistent viral productive replication and acquire morphologic and functional features of KS spindle cells, reinforcing the hypothesis that EPCs may represent putative precursors of these cells.

HPC 02. miRNA-148a in HBx Associated Liver Cancer

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Background: The hepatitis B virus encoded X antigen, or HBx, is a trans-regulatory protein that alters the activity of selected transcription factors and cytoplasmic signal transduction pathways. HBx up-regulates the expression of a unique gene, URG11, which in turn up-regulates catenin-catenin, thereby contributing importantly to hepatocarcinogenesis.
Methods: HepG2 cells stably expressing HBx (HepG2X), URG11 (HepG2-URG11) or the bacterial chloramphenicol acetyltransferase (CAT) gene (HepG2CAT) were subjected to microRNA array analysis for more than 1000 human miRNAs.
Results: HBx and URG11 alter the expression of multiple microRNAs by miRNA array analysis, and among these, miR-148a was found to be up-regulated. miR-148a was also elevated in many HBV-infected liver samples from patients. To study the function of miR-148a, HepG2 (hepatoblastoma) and Hep3B (hepatoma) cells expressing HBx or URG11 were transduced by recombinant lentiviruses encoding anti-148a. Anti-miR-148a suppressed cell proliferation, viability, cell cycle progression, cell migration, and anchorage independent growth in soft agar. β-catenin levels were decreased (by western blotting) in anti-miR148a cells expressing HBx or URG11.
Conclusions: Thus, miR-148a may play a central role in HBx/URG11 mediated HCC, and may be an early diagnostic marker and/or therapeutic target associated with this tumor type.

IMMUNITY AND INFLAMMATION

IM 01. Selective Induction of VEGF-A by TLR4 and TLR8 in Human Dendritic Cells

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Background: Angiogenesis is an essential process in tissue repair, foetal development and female reproductive cycle and is also associated with many pathologic conditions. In previous work we have reported that LPS has the ability to induce biological active VEGF-A in dendritic cells (DC).
Methods: DC were stimulated with different TLR ligands and the production of VEGF-A and PGE2 were evaluated by ELISA.

Results: In the present study we report that LPS (TLR4) and R848 (TLR8) have the peculiar ability to induce VEGF-A production. This action relies on the unique ability of TLR4-8 to induce the release of PGE2 in DC. This activity is dependent on the activation of NFkB and MAPKs and relies on the autocrine activation of EP2 and EP4, two of the PGE2 receptors. The lack of activity of the other TLR ligands, namely PAM (TLR1/2), FSL-1 (TLR2/6), Poly I:C (TLR3), Flagellin (TLR5) and Imiquimod (TLR7) was dependent on their inability to produce PGE2. This defect was due to the lack of activation of cPLA2, as in the case of PAM and FSL-1, or to the failure of Poly I:C, Flagellin and Imiquimod to activate COX-2 transcription.
Conclusions: These data indicate that PAMPs, DAMPs and alarmins may directly activate a pro-angiogenic program in DC through the activation of TLR4 and TLR8, or by the interaction with other TLRs in the presence of the synergic action of PGE2 produced within the inflammatory microenvironment.

IM 02. Syk Kinase Activity Regulates Endocytosis of Ubiquitinated FcεRI on Mast Cells

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Background: The aggregation of the high affinity IgE receptor (FcεRI) on mast cells allows the activation of the tyrosine kinase Syk, that plays a central role in the propagation of intracellular signals. Concurrently, Syk activates the ubiquitin ligase c-Cbl that, in turn, promotes ubiquitination and down-regulation of engaged FcεRI. The aim of this study is to better investigate the role of Syk in regulating antigen-induced FcεRI.
Methods: By using a rat leukemia mast cell line, we performed siRNA knock down of Syk. This approach was combined with flow cytometry, fluorescence microscopy and biochemical analysis.
Results: We demonstrated that Syk depletion affects FcεRI internalization and prevents the delivery of aggregated receptors to lysosomes. We also found that Syk activity is required to promote phosphorylation and monoubiquitination of Hrs, an ubiquitin-binding protein directly involved in the delivery of ubiquitinated receptors to lysosomes for degradation. We are currently investigating whether Hrs phosphorylation and ubiquitination could affect protein localization, stability and/or alter Hrs ability to bind molecules that act further along the endocytic pathway.
Conclusions: In summary, we provide evidence that Syk, commonly known as a positive regulator of FcεRI-mediated signal transduction, is also capable of limiting FcεRI-triggered signals by regulating the endocytosis of ubiquitinated receptors. Syk-dependent Hrs tyrosine phosphorylation and ubiquitination is required to drive a proper FcεRI endocytosis, thus targeting activated receptor complexes to lysosomes for degradation. Elucidating the mechanisms responsible for FcεRI down-modulation may be critical for the development of novel therapeutic strategies in the treatment of disorders that involve mast cell activation.

IM 03. SR141716 Reduces Keratinocyte Growth by Induction of Apoptosis and Exerts Topical Anti-Inflammatory Activity in Mice

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Background: Emerging findings have demonstrated that the cannabinoid receptor CB1 antagonist, rimonabant (SR141716) exerts potential anti-proliferative and anti-inflammatory action. The aim of this study is to assess the effects of SR141716 *in vitro* in murine immortalized keratinocytes and *in vivo* by evaluation of the topical anti-inflammatory activity.
Methods: Keratynocyte viability and death have been performed by trypan blue exclusion assays, cell cycle progression has been assessed by flow cytometry and the expression of apoptotic and anti-apoptotic markers, cyclins, pathways of signal transduction and CB1 receptor levels, have been evaluated by western blot. The topical anti-inflammatory properties of SR141716 have been analysed by inhibition of Croton oil-induced ear dermatitis in mice.
Results: We found that SR141716 reduces cell viability and induces apoptosis as revealed by the enhanced number of cells in the subG0 phase of the cell cycle, the expression of the apoptotic marker Bax and the reduced levels of the anti-apoptotic Bcl-2 and xIAP. In addition, reduced levels of both phosphorylated Akt and NFκB were detected associated with regulation of the expression of total NFκB and IkBα, phosphorylated IkBα, cyclins D1, E and A. We found that the CB1 receptor is modulated by SR141716. In Croton oil-induced ear dermatitis, SR141716 significantly reduces edema and leukocyte infiltrate.
Conclusions: We demonstrated that SR141716 reduces cell growth, inducing cell death in abnormal proliferating immortalised keratinocytes, and decreases Croton oil-induced ear dermatitis in mice. Our findings suggest the potential application of SR141716 as a topical anti-inflammatory drug.

IM 04. Naringin Dry Powder Inhalers Reduce Cystic Fibrosis Intrinsic Inflammation *In Vitro*

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Background: Cystic Fibrosis (CF) is an autosomal genetic disorder caused by mutations in the gene encoding the CF Transmembrane Conductance Regulator (CFTR) protein. Defective CFTR function induces the expression of proinflammatory mediators also in absence of any infection. MAPK/ERK and NFκB pathways are intrinsically overactivated in CF cells. Current CF therapy is directed to reduce excessive inflammation in order to delay lung tissue damage. Flavonoids like Naringin (N) have been proposed as promising antinflammatory drugs. Therefore we developed N- Dry Powder Inhalers (N-DPIs) as pulmonary formulations and tested their ability to reduce CF intrinsic inflammation in comparison to neat drug.
Methods: Dry powders were prepared by co-spray-drying the drug and a series of aminoacids. Morphology, size, density, dissolution rate were evaluated and correlated to process parameters. The aerodynamic properties were assessed by both a Single Stage Glass Impinger and an eight-stage Andersen cascade impactor using a Turbospin®. The neat compound and the optimal formulation were tested in CF bronchial epithelial cells (CuFi) by analyzing the expression levels and phosphorylation status of the key enzymes of the MAPK/ERK and NFκB pathways.
Results: Our results indicate that N co-sprayed with leucine is able to inhibit both the expression levels of IKKα and IKKβ and to reduce the phosphorylation of the direct NFκB inhibitor IκBα. Moreover, N and N-DPIs also inhibited the phosphorylation of ERK1/2 kinase.
Conclusions: In conclusion, these data suggest that through the technological handling increasing immediate solubility and decreasing cohesiveness of the dry-powders, N-DPIs formulations could be proposed as preventive treatment in CF disease.

IM 05. Dendritic Cells/Natural Killer Cross-Talk: A Novel Target for Human Immunodeficiency Virus Type-1 Protease Inhibitors

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Background: HIV-1 Protease Inhibitors, namely PIs, originally designed to inhibit HIV-1 aspartic protease, can modulate the immune response by mechanisms largely unknown, and independent from their activity on viral replication. Here, we analyzed the ability of PIs to interfere with differentiation program of monocytes toward dendritic cell (DCs) lineage, a key process in the inflammatory response.
Methods: Monocytes from healthy donors were isolated and induced to differentiate in vitro in the presence or absence of saquinavir, ritonavir, nelfinavir, indinavir or amprenavir (sqv, rtv, nlfv, idv, apv).
Results: These drugs demonstrated a differential ability to sustain the generation of immature DCs (iDCs) with an altered phenotype, including low levels of CD1a, CD86, CD36 and CD209. DCs generated in the presence of rtv failed to acquire the typical phenotype of mature DCs (mDCs), and secreted lower amounts of IL-12 and IL-15. Accordingly, these aberrant mDCs failed to support activation of autologous Natural Killer cells, and resulted highly susceptible to NK cell-mediated cytotoxicity.
Conclusions: Our findings uncover novel functional properties of PIs within the DC-NK cell cross-talk, unveiling the heterogeneous ability of members of this class drugs to drive the generation of atypical monocyte-derived DCs showing an aberrant phenotype, a failure to respond appropriately to bacterial endotoxin, a weak ability to prime autologous NK cells, and a high susceptibility to NK cell killing. These unexpected properties might contribute to limit inflammation and viral spreading in HIV-1 infected patients under PIs treatment, and open novel therapeutical perspectives for this class drugs as immunomodulators in autoimmunity and cancer.

IM 06. Affinity Maturation of Antibodies in the 2-Phenyl-5-Oxazolone System

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Background: Affinity maturation of antibodies is the process whereby more efficient antibodies are produced through somatic hypermutation and antigen-guided selection. No extensive study is available at the moment concerning the relationship between somatic mutations and their structural counterpart. The antibody response to 2-phenyl-5-oxazolone has been thoroughly investigated from the genetic point of view. It consists of three antibody classes, with each member of each class derived from a unique pair of VH and VL germline genes by somatic hypermutation. In this project, we are investigating the structure of the VH and VL domains of 10 representative antibodies.
Methods: The VH and VL domains of each antibody are being expressed as recombinant scFvs, crystallised, and their structure determined by X-ray crystallography.
Results: Our investigation has led so far to the determination of 4 antibody structures and has shown that in class III antibodies the increase in affinity is mainly determined by the improvement in the surface complementarity of the hapten binding site, while a low-

affinity class I antibody structure suggests that in this class it depends on a fine reorganisation of the binding site involving both charge changes and surface shape modifications. We have recently solved the structure of a high affinity class II antibody, suggesting a maturation strategy based on the increase of surface and charge complementarity of the binding site, and on the introduction of a specific bond with the oxazolone ring.
Conclusions: These results are relevant to determine the principles underlying affinity maturation of antibodies.

IM 07. Mixed TH1 and TH17 Cell Responses in Murine Granulomatous Experimental Autoimmune Thyroiditis Induced by Hydrophobic Intermediates of Thyroglobulin Dissociation/Unfolding in Urea

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Background: A granulomatous experimental autoimmune thyroiditis (G-EAT), with follicle lysis, hystiocytic/granulocytic infiltration and fibrosis, was induced in CBA/J(H-2k) mice by immunization with hydrophobic intermediates of the dissociation/unfolding of human thyroglobulin (hTg) in urea, namely, partially unfolded hTg monomers (PUM) and non-natively reassociated dimers (NND).
Methods: Female CBA/J(H-2k) mice were immunized with native hTg, partially unfolded monomers (PUM) or non-native dimers (NND), obtained by denaturation of hTg in 3.5 M urea at pH 9.0, and separated by sucrose density centrifugation. On day 18, we evaluated thyroid histology, splenocyte proliferation and multiple cytokine levels in the supernatants, by fluorescent bead flow cytometry.
Results: Proliferative responses of splenocytes from mice immunized with PUM and NND to the latter antigens in vitro were stronger than those of mice immunized and restimulated with native hTg. They were marked by the production of higher levels of IFN-γ, TNF-α, IL-2, IL-6 and IL-10, compared with native hTg, and by a unique IL-17 response. Significant cytokine production occurred also with control splenocytes exposed to PUM and NND, which points to the participation of APCs and NK cells in the effects of unfolded hTg.
Conclusions: The greater ability of partially unfolded hTg species with increased hydrophobicity to induce autoimmune thyroid damage, with respect to native hTg, seemingly relied upon their ability to induce a stronger TH1 and a unique TH17 cell response. Further investigation is warranted regarding human AITD forms possibly triggered by hTg folding/dimerization defects, or by the release of unfolded, nascent hTg molecules upon thyroid cell damage.

IM 08. B7h Triggering Inhibits Umbilical Vascular Endothelial Cell Adhesiveness to Colon Carcinoma Cell Lines and Polymorphonuclear Cells

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Background: ICOS is a receptor expressed by activated T cells and delivers costimulatory signals modulating TH cell activation. It binds B7h expressed by cells of both haematopoietic and non-haematopoietic origin including vascular endothelial cells (EC), which are key players in leukocyte recruitment into tissues and metastatic dissemination of tumor cells.
Methods: This work assessed the effect of B7h triggering by a soluble form of ICOS (ICOS-Fc) on the adhesion of colon carcinoma cell lines to human umbilical EC (HUVEC).
Results: We found that B7h triggering inhibited HUVEC adhesiveness to HT29 and DLD1 cells (by 50 and 35% respectively), but not to HCT116 cells. The effect was dependent on the ICOS-Fc dose, detectable as soon as 30 min after treatment and still present after 24 hrs. HUVEC treatment with ICOS-Fc did not modulate expression of adhesion molecules and cytokines, but substantially down-modulated phosphorylation of p38 and ERKs induced by E-selectin triggering, which has been shown to increase HUVEC adhesiveness to colon carcinoma cell lines possibly by modulating the spatial organization of EC adhesion molecules. Moreover, treatment with ICOS-Fc also inhibited HUVEC adhesiveness to polymorphonuclear cells (PMNs) (by 50%) which also involves E-selectin.
Conclusions: This work shows that the B7h:ICOS interaction may modulate spreading of cancer metastases and recruitment of PMNs in inflammatory sites, which opens a novel view on the use of ICOS-Fc as an immunomodulatory drug.

IM 09. ICOS Triggering Induces IL17 Secretion in Human Naive T Helper Cells

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Background: ICOS is a T cell costimulatory molecule belonging to the CD28 family and binds B7h belonging to the B7 family. We previously showed that, in human naive Th cells, ICOS triggering elicited Th1 cells in the presence of IL-2 and Treg cells in its absence. Human Th17 cells are characterized by IL-17A, IL-17F, IL-22, IL-21 and IL-26 expression, but no data are available about the role of ICOS on these cells.
Methods: This work evaluated, in human naïve CD4+ T cells, the effect of ICOS triggering by a soluble form of B7h (B7h-Fc) in Th17 differentiation.
Results: We found that ICOS costimulation induced high levels of IL-17A and low levels of IL-17F in CD3-stimulated naive Th cells cultured in the presence of exogenous TGF-1β+IL-1β, and this effect was increased by further addition of IL-6, IL-21, or IL-23. By contrast, CD28 costimulation induced low levels of IL-17A and high levels of IL-17F. Moreover, ICOS costimulation induced expression of both RORC2 and RORA, which are transcription factors involved in Th17 cell differentiations.
Conclusions: These data suggest that ICOS costimulation favours differentiation of Th17 cells which are partly different than those induced by CD28 costimulation in terms of secretion of IL17A and IL-17F. This may have an impact on several processes, such as angiogenesis and autoimmune responses, in which these cytokines seem to have a different impact.

IM 10. Role of Tissue Inhibitor of Metalloproteinase-1 In Development of Autoimmunity Lymphoproliferation

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Background: Inherited defects decreasing function of the Fas death receptor cause the autoimmune lymphoproliferative syndrome (ALPS) with autoimmunities, spleen and lymph node enlargement, and expansion of TCRα/β+ CD4/CD8 double negative T cells; this expansion is absent in an ALPS variant named Dianzani Autoimmune Lymphoproliferative Disease (DALD). A cDNA array analysis of peripheral blood mononuclear cells from a DALD patient detected overexpression of osteopontin (OPN) and Tissue Inhibitor of metalloproteinases 1 (TIMP-1). Previous work on OPN had detected increased serum levels in DALD patients, associated with variations of its gene.
Methods: TIMP-1 levels were evaluated by ELISA in sera and culture supernatants, and by Real Time PCR in cell lysates. The TIMP-1 gene was sequenced from genomic DNA. Activation- (AICD) and Fas-induced (FICD) cell death were induced in vitro using anti-CD3 and anti-Fas antibodies respectively.
Results: ELISA evaluation of TIMP-1 in sera from 11 ALPS and 21 DALD patients, and 50 healthy controls detected higher levels in both patients groups ($p<0.0001$), unassociated with variations of the TIMP-1 gene. Since both patients' groups displayed increased levels of OPN too, we investigated whether OPN influences TIMP-1 expression in vitro in peripheral blood mononuclear cells. Results showed that OPN increased TIMP-1 secretion in a time- and dose-dependent manner and this effect was mainly exerted on onocytes. Moreover, TIMP-1 significantly inhibited both FICD and AICD of lymphocytes.
Conclusions: These data suggest that the high OPN levels may support high TIMP-1 levels in ALPS and DALD, and hence worsen the apoptotic defect in these diseases.

IM 11. KLRG1+ NK Cells Include Two Distinct Populations Lined by CX3CR1 Expression

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Background: Expression of chemotactic molecules in the bone marrow (BM) microenvironment may deeply influence NK cell maturation by controlling their positioning into specific niches. In the present study, we functionally characterized CX3CR1 expressing NK cell subsets.
Methods: CX3CR1 expression was assessed by FACS analysis and the functions of NK cells by migration, IFN-gamma intracellular staining and citotoxicity assays.
Results: Using CX3CR1+/GFP mice, we showed that CX3CR1 is prevalently expressed by the KLRG1+ NK cell subset that is considered a quiescent and terminally differentiated NK cell population with reduced effector functions. Within KLRG1+ NK cells, CX3CR1+ cells evidenced reduced CXCR4 expression and function that was associated with different positioning within BM as compared to the CX3CR1- NK cells. In addition, CX3CR1+ NK cells displayed impaired capability to produce IFN-gamma and to lyse YAC-1 target cells. By means of adoptive transfer experiments we observed that CX3CR1 expression can be acquired by KLRG1+/CX3CR1- NK cells, while KLRG1+/CX3CR1+ NK cells stably expressed CX3CR1. We also analyzed the role of CX3CR1 during NK cell maturation, comparing NK cell tissue distribution in heterozygous CX3CR1+/GFP and in CX3CR1-deficient CX3CR1GFP/GFP mice. We observed tissue accumulation of GFP+ NK cells in CX3CR1GFP/GFP mice that was associated to an

increased number of BM NK cells during steady state and to a defective BM NK cell mobilization during POLY I:C –induced inflammation.
Conclusions: All together, our findings show that CX3CR1 is a marker of a KLRG1+ NK cell subset with impaired effector functions that can irreversibly differentiate from the CX3CR1- cells under steady state conditions.

IM 12. Lymphocyte Signaling Activation Regulates Expression of PRDM Genes.

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Background: The PRDM family genes play a key role in controling cellular processes, such as cell cycle progression, control of development and thomeostasis.Generally, PRDM genes express molecular variants generated by alternative splicing or alternative use of different promoters. The imbalance between the two forms (PR-plus and PR-minus) is found in many human tumors, particularly in leukemias and lymphomas. In these diseases, the PR-minus form is more expressed than PR-plus form with tumour suppressor activities.
Methods: The qPCR was performed on human lymphocytes cDNA. Polyclonal activation was performed with: PMA/ionomycinA, anti-CD3/anti-CD28 antibodies, trasduction PI3 kinase and MEK1/2 pathways inhibitors (LY294002, Wortmannin and PD98059).
Results: Our data showed the lymphocyte activation effects on each PRDM gene and on the gene expression variants of the same gene. PRDM1 and PRDM2 genes are susceptible to polyclonal activator PMA/ionomycinA within a few hours, with an increase in the expression levels of their PR-plus form, as well as the Immediate Early Growth Response Genes. The PRDM genes PRDM1-17in both T and B lymphocytes were studied after specific polyclonal activation. PRDM1, considered a master regulator of lymphocyte differentiation, and PRDM2 were inhibited by Wortmannin. The other members of the PRDM gene family show a different behavior among themselves and a different regulation of their alternative forms.
Conclusions: As described before, in cancer models, PRDM2 gene has shown a ying-yang paradigm even in the physiological model, the lymphocyte, which is directly attributable to the fundamental TCR/CD28 costimulation signals. A similar model can be supposed for other PRDM genes.

IM 13. Development, Surface Phenotype and Function of Human Interleukin 17–Producing Vγ9Vδ2 T Cells

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Background: In healthy adults, the major peripheral blood γδ T-cell subset expresses the Vγ9Vδ2 TCR and displays pleiotropic features in immune response. Although the transitional γδ T cell response is widely viewed as pro-inflammatory and/or cytolytic, there is increasing evidence that different microenvironments drive distinct effector functions, similarly to CD4 αβ helper T cells.
Methods: Culture of Vγ9Vδ2 T cells with dendritic cells and phosphoantigens, in the presence of a cocktail of cytokines (IL1-β, TGF-β, IL-6 and IL-23) leads to selective expression of the transcription factor RORc and differentiation of IL-17 producing Vγ9Vδ2 T cells.
Results: We determined the phenotype, intracellular cytokine content, the gene profile expression for RORc and the ability of soluble factors released following co-colture of these cells with DC to induce leukocyte migration and phagocytic activity. The vast majority of IL-17+ Vγ9Vδ2 cells had a CD27-CD45RA+ phenotype and typically expressed the CCR6 chemokine receptor, but lacked CCR5, CXCR3 and CXCR5 receptor expression. They also expressed TRAIL, FasL and granzyme B, but not perforin, and displayed potent cytolytic activity toward epithelial tumor cell targets. Moreover, upon antigen stimulation in vitro, IL-17+ Vγ9Vδ2 cells released unknown soluble factors which enhanced chemotaxis and phagocytic activity of leukocytes, as well as induced the release of β-defensin when cultured with epithelial cells.
Conclusions: These preliminary results highlight the role of IL-17+ Vγ9Vδ2 cells T as new players of immunoregulation and protective immune responses.

IM 14. Modulation of J774A.1 Macrophages Activity by Different Mycotoxins.

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Background: Mycotoxins such as deoxynivalenol, nivalenol, aflatoxin B1, aflatoxin B2 and their hydroxylated metabolites, aflatoxin M1 and aflatoxin M2, respectively are secondary metabolites produced by microfungi such as *Fusarium* and *Aspergillus* spp. Mycotoxins are often contaminants of food and feedstuffs particularly vegetables and derived products but also products of animal origin (Hussein HS and Brasel JM, Toxicology, 2001,167:101-134). Many toxic effects are ascribed to mycotoxins and some of them are immunotoxic agents (Marzocco S et al, Toxicol.Lett, 2009,189:21-26) so the aim of the current study was to evaluate the effects of NIV, DON, AFM1 and AFM2, alone or in combination, on J774A.1 macrophages.
Methods: Cell viability was evaluated on cultured J774A.1 macrophages exposed to serial dilutions of mycotoxins, alone or in combination, for 24,48,72hours through MTT assay. The activation of macrophages was evaluated by measuring the NO2- production in medium of cells exposed to mycotoxins and stimulated with LPS for 24 hours. Protein expression was evaluated on cell lysates by Western blot analysis.
Results: Results showed that both trichothecenes significantly induced apoptosis in cultured macrophages in a concentration-dependent manner; NIV showed a stronger pro-apoptotic effect than DON. No interactive effects were observed between NIV and DON. AFM1 and AFM2 did not affect cells viability; nevertheless, they reduced nitrite production only at the highest concentration.
Conclusions: In conclusion the mycotoxins evaluated in the current study can interfere in immunomodulation at different levels. Further investigations will be necessary for better clarifying how these mycotoxins interfere in immuno-competent cell functions.

IM 15. Magnesium Deficiency Promotes a Proatherogenic Phenotype in Cultured Human Endothelial Cells.

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Background: Phenotypic modulation of endothelium to a dysfunctional state contributes to the pathogenesis of atherosclerosis, partly through the activation of the transcription factor NFkB. Several data indicate that magnesium (Mg) deficiency caused by poor diet and/or errors in its metabolism may be a missing link between diverse cardiovascular risk factors and atherosclerosis.
Methods: Human umbilical vein endothelial cells (HUVEC) were utilized and cultured in low (0.1 mM) or normal (1.0 mM) extracellular Mg. NFkB activation was evaluated by various techniques, including electrophoretic mobility shift assay and luciferase activity. Secreted cytokines and growth factors were investigated by protein array and results confirmed by western blot or ELISA. Matrix Metalloproteases (MMP) activity was evaluated by zymography.
Results: We found that HUVEC cultured in low Mg rapidly activate NFkB, an event which is prevented by exposure to the anti-oxidant Trolox. NFkB activation correlates with marked alterations of the cytokine network in HUVEC in low Mg. In particular, RANTES, interleukin 8 and platelet derived growth factor BB, all important players in atherogenesis, were upregulated. We also describe the increased secretion of metalloproteases-2 and -9 and of their inhibitor TIMP-2. Interestingly, by zymography we show that gelatinase activity predominated over TIMP-2.
Conclusions: These results indicate that low Mg promotes endothelial dysfunction by inducing pro-inflammatory and pro-atherogenic events.

IM 16. The Effects of β-Glucan on Sepsis

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Background: β-glucans, glucose polymers of the fungal cell wall, stimulate immune cells. The soluble β-glucan, PGG-glucan, stimulates without inducing cytokines. The prophylactic effects of PGG-glucan have been evaluated, however, it is unknown if PGG-glucan can benefit a host with an ongoing infection. We evaluated the effects of PGG-glucan on the host response to polymicrobial sepsis when administered after the onset of infection.
Methods: CD-1 mice were administered 10mg/kg PGG-glucan or the polysaccharide control, dextran, one hour after cecal ligation and puncture (CLP). Six hours after CLP, blood was obtained from the tails of mice, and cytokine levels measured by ELISA. Survival was monitored over 10 days. Liver and blood samples were obtained from additional mice 24 hours after CLP and bacteremia was monitored.
Results: PGG-glucan enhanced 10-day survival in female mice, but not in male mice. Elevated IL-6 levels predicted mortality, however, differences in IL-6 values determined to predict 100% mortality between the sexes existed. PGG-glucan did not enhance 10-day survival in ovariectomized female mice or in ovariectomized female mice given a dose of estrogen, indicating that hormones are involved in the protection mediated by PGG-glucan, but that estrogen is not sufficient for protection. PGG-glucan reduced IL-6 and IL-10 levels in male and female mice and the CLP-induced bacterial growth in female mice.
Conclusions: The development of PGG-glucan as a therapeutic agent could aid septic patients. This is the only study to examine the effects of PGG-glucan on sepsis when administered as a single dose after injury.

IM 17. Autoantibodies in Multiple Autoimmune Syndromes

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Background: The multiple autoimmune syndromes (MAS) form different clusters of autoimmune disorders and are endocrinopathies characterized by the coexistence of at least two autoimmune mediated diseases. According to a widely diffuse classification, 4 main types of MAS (type 1, type 2, type 3 and type 4) are described. The presence of

specific autoantibodies in patients with an autoimmune endocrinopathy define the incomplete MAS.
The aim of this review is to focus on the frequency of autoantibodies on the MAS type III, the most diffuse form of these endocrinopathies, and on the important diagnostic role of different types of autoantibodies in the prediction of the onset of the second autoimmune disease in patients with autoimmune thyroid diseases. The author provides an analysis of the utility of such autoantibodies as markers of the underlying disorders in MAS IIIB, particularly in the case of autoimmune gastritis (thyrogastric autoimmunity) and celiac disease.
Conclusions: The introduction of new multiplex proteomic technologies in autoimmunity laboratories, based on the use of microarrays, represents a fundamental tool to analyze simultaneously the concentrations of specific autoantibodies and to define same autoantibodies as predictive markers of disease.

IM 18. 'Serological Biopsy' in the Diagnosis of Autoimmune Gastritis
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Background: Evaluating clinical efficiency of determination of antiparietal cell (APCA), antiintrinsic factor (anti-IF) antibodies and gastrin, serological biopsy, in the diagnosis of autoimmune gastritis (AIG).
Methods: We searched 181 patients (aged 25-81) affected by iron deficiency (IDA) (39.8%) or vitamin B12 anemia (60.2%), clinical conditions related to AIG. We determined APCA, anti-IF by quantitative ELISA (Aesku) and gastrinemia with ICMA (Siemens).
Results: 83 patients showed APCA, 14 of them also had anti-IF. Gastrinemia had significantly increased in 44 positive APCA patients but was standard in negative subjects. 83 APCA and 11 seronegative patients were subjected to gastroscopy. Histological and serological biopsy has led to stratification of patients into 4 groups: 1) 30 patients (average age: 53 ± 20) with AIG showed APCA and hypergastrinemia 2) 14 subjects (70 ± 16) with atrophic gastritis had APCA, anti-IF as well as hypergastrinemia 3) 18 patients (46 ± 12) with nonspecific lymphocytic gastritis showed APCA, normal gastrinemia, anti-IF was absent 4) 21 borderline APCA patients (71 ± 12) had anti *Helicobacter pylori* (Hp) antibodies, normal gastrinemia and were affected by Hp multifocal atrophic gastritis. Finally the 11 seronegative individuals showed superficial gastritis
Conclusions: "Serological biopsy" has a high predictive value for AIG and represents a diagnostic laboratory profile for people affected by IDA refractory to oral iron treatment or vitamin B12 dependent.

MICROENVIRONMENT-DRIVEN TUMOR PROGRESSION

MTP 01. Cannabinoid CB1 Receptor is a Novel Target for Angiogenesis Inhibition: Genetic and Pharmacological Evidence
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Background: In the present study we investigated *in vitro* and *in vivo* the role of CB1 receptor signaling in angiogenesis and the therapeutic exploitation of CB1 blockade as an anti-angiogenic strategy. The study arises from the observation that CB1 receptor expression is induced during angiogenesis in endothelial cells.
Methods: In order to define the specific role of CB1 receptor signaling during the angiogenic process, two different strategies have been carried out: genetic and pharmacological inactivation of CB1 receptors.
Results: Silencing CB1 by RNA interfering or blocking its signaling with a selective antagonist (SR141716) resulted in a dose-dependent inhibition of bFGF-induced endothelial cell proliferation, migration and capillary-like tube formation through the pro-survival and migratory pathways, p42/44 MAPK and FAK/PI3K/AKT. By inhibiting RhoA activation CB1 blockade inhibited the matrix metalloproteinase-2 secretion strongly involved in cell migration. To corroborate the potential therapeutic exploitation of CB1 blockade as an anti-angiogenic strategy, we performed several in vivo assays founding that CB1 blockade was able to inhibit in vivo bFGF-induced neovascular growth in the rabbit cornea assay. A relevant finding was the ability to reduce ocular pathological neo-vascularization in mouse oxygen-induced retinopathy.
Conclusions: These results demonstrate that CB1 signaling is involved in angiogenesis and that could be a target for treatment of diseases where excessive neoangiogenesis is the underlying pathology.

MTP 02. The Mutant K303R Estrogen Receptor α (ERα) as an Amplifier of the Crosstalk Between Leptin and Estrogen Signaling Pathway in Breast Cancer.
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Background: Despite the success of hormonal therapy with aromatase inhibitors (AIs), many patients exhibit de novo resistance. We identified a lysine to arginine transition at residue 303 (K303R) within ERα in premalignant and invasive breast cancers, which confers estrogen-hypersensitivity and resistance to the AI anastrazole via enhanced cross-talk with IGF1-R/PI3K/Akt-signaling. Microarray experiments showed increased leptin receptor mRNA levels in K303R-expressing MCF-7 breast cancer cells. The adipose surrounding stromal cells and adipokines, as leptin, strongly influence the phenotypic behavior of malignant breast epithelial cells. We hypothesized that the K303R-mutation may potentiate ERα's role as effector of leptin signalling, thus enhancing cell proliferation, and invasiveness, resulting in a more aggressive phenotype of K303R-associated cancers.
Methods: We employed microarray analyses, realtime PCR and immunoblotting analysis to evaluate leptin receptor expression. Signaling pathways were analyzed by immunoblotting analysis.We used anchorage-dependent and-independent growth assays.
Results: We found, in mutant-expressing cells, an increase in the leptin receptor isoforms, and elevated phosphorylation of MAPK, JAK2/STAT3 and PI3K/Akt signaling-molecules with leptin treatment. Post-translational modifications of ERα, such as phosphorylation, tightly regulate its function. Leptin treatment enhanced phosphorylation of K303R-ERα at serine-167 and-118, and concomitantly mutant receptor transcriptional activity. The mutant cells also showed an increased proliferation rate after leptin treatment.
Conclusions: Since the K303R mutation was identified in 30% of typical hyperplasia, we could speculate that the pressure of the microenvironment in the presence of this specific mutation hypersensitive to leptin signaling may promote or accelerate the development of cancers from premalignant breast lesions, further increasing risk in obese women.

MTP 03. Serum Adipokine Levels In Colorectal Cancer Patients With and Without Synchronous Distant Metastases
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Background: Obesity is associated with a state of chronic low level of inflammation, characterized by abnormal cytokine production and the activation of pro-inflammatory signaling pathways. Adiponectin and leptin are the most abundant adipocytokines produced by adipocytes. They seem also to have a modulatory role in the regulation of proliferation and migration of tumor cells. Aim. To evaluate whether alterations of serum levels of adiponectin and leptin in colorectal cancer patients are associated with the presence of metastases.
Methods: Seventy-nine consecutive patients (46 males and 33 females, mean age 65.6 ±8.1 years) with histologically proven colorectal cancer were enrolled in the study. Serum adiponectin and leptin levels were measured using an ELISA assay (BioVendor Res, Czech Rep. and Diagnostics Biochem Canada Inc.).
Results: Mean adiponectin levels were lower in patients with metastases compared with non-metastases patients but the differences reached only borderline statistical significance (0.52 ± 0.02 vs 0.56 ± 0.01, mean ± SE, P= 0.07, Unpaired t-test). No difference in leptin levels was present between two groups of patients. Analysis of mean levels of leptin:adiponectin ratio (LAR) revealed lower levels among patients with metastases compared with non-metastases patients, but the differences did not reach statistical significance (16.9 ± 2.9 vs 20.7 ± 3.2, mean ± SE, P= 0.2, Unpaired t-test).
Conclusions: More studies and a larger number of patients are need to substantiate associations between the presence of metastases and serum adipokines abnormalities in colorectal cancer patients.

MTP 04. The Upregulation of GPR30/GPER by Hypoxia is Involved in the Antiapoptotic Effects Exerted by Estrogens in Breast Cancer Cells
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Background: Estrogens play a pivotal role in the development of breast cancer through a complex signaling network. Recent studies have suggested that GPER, also known as GPR30, mediates rapid effects induced by estrogens in diverse normal and cancer tissues. Hypoxia is a common feature of solid tumors where it promotes relevant biological responses leading to apoptosis, cell survival and cancer progression. The response to low oxygen environment is mainly mediated by the hypoxia-inducible factor named HIF-1α, which triggers a signaling pathway involved in the adaptative mechanisms activated in tumor cells.
Methods: Cloning, Transfection experiments, RT-PCR, immunoblotting, ChiP assay, gene silencing, Tunnel assay.

Results: We demonstrate that HIF-1α mediates the up-regulation of GPER induced by the hypoxia-mimetic agent $CoCl_2$ in estrogen receptor (ER)-negative SkBR3 breast cancer cells. As assessed by transfection experiments and ChIP assays, HIF-1α responsive elements located within the promoter region of GPER were shown to be involved in the hypoxia-dependent GPER transcription. Furthermore, we found that ROS-mediated activation of EGFR/ERK signaling contributes to the GPER induction upon $CoCl_2$ treatment. Interestingly, the apoptotic response to hypoxia was prevented by estrogens through GPER in SKBr3 cells, as demonstrated using gene silencing experiments.
Conclusions: Taken together, our data suggest that the hypoxia-induced expression of GPER, which mediates the anti-apoptotic effects elicited by estrogens in a low oxygen microenvironment, may be considered as an additional molecular mechanism involved in breast cancer progression.

MTP 05. Oncostatin M Triggers EMT In Human HepG2 Cells

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Background: Oncostatin M (OSM), a cytokine that belongs to the interleukin-6 family, has been reported to orchestrate hypoxia-influenced processes such as liver development,regeneration and angiogenesis, being then potentially involved in the progression of cirrhosis and hepatocellular carcinoma. Accordingly, OSM has been found to be overexpressed in cirrhotic liver. Recently, hypoxia, as an independent signal, has been shown to induce epithelial-to-mesenchymal transition (EMT) in cancer cells, including HepG2 cells. In this connection, OSM-related signaling pathway has been reported to up-regulate HIF1α. This study has been designed in order to investigate whether OSM may act as a stimulus able to induce EMT in HepG2 cells.
Methods: EMT, invasiveness and signal transduction pathways were analysed in HepG2 cells, treated with OSM 10 ng/ml.
Results: OSM induces EMT changes in HepG2 cells within 48-72 hrs: nuclear translocation of SNAI1 and SNAI2, E-cadherin down-regulation, overexpression of α-SMA and expression of matrix metalloprotease-2 (MMP2). Moreover, OSM induced in HepG2 a significant up-regulation of HIFs as well as stimulation of invasiveness. Exposure of HepG2 cells to OSM is followed by a rapid increase in intracellular levels of ROS, early phosphorylation/inactivation of GSK-3β and phosphorylation of relevant kinases: PI-3K, ERK1/2, SAPKs (JNK1/ 2 and p38 MAPK) and STAT3. Experimental manipulations suggest a direct link between activation of selected kinases (ERK1/2, PI3K, JNK1/2) or increase in ROS and induction of invasiveness.
Conclusions: OSM is able to induce EMT in human HepG2 cells and to stimulate their increased invasiveness through the involvement of ROS and critical kinases involved in signal transduction.

MTP 06. Roles of Mast Cells and their Mediators in Human Thyroid Carcinomas

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Background: In different human carcinoma types mast cell infiltrate is increased with respect to normal tissue and mast cell density correlates with a poor prognosis.
Methods: To assess the presence of mast cells in human thyroid cancer, we used IHC on human thyroid carcinoma samples. To rest the effect of mast cells on thyroid cancer cells, we used DNA synthesis, apoptosisand invasion assays. We also used tumor growth assay in nude mice.
Results: We found that human thyroid carcinomas feature a remarkable mast cell infiltrate whose intensity correlates with the invasive phenotype. Using chemoattraction assays, we show that thyroid cancer cells attract mast cells through the release of VEGF-A. The injection of HMC-1 mast cells into the tail vein of nude mice caused their recruitment to thyroid carcinoma cell-induced xenografts. Thyroid cancer cells stimulate histamine release and cytokine synthesis in human mast cells. Mast cell-released mediators enhanced the proliferation, survival and invasive behavior of thyroid cancer cells in vitro. Moreover, mast cells promote the growth of thyroid carcinoma xenografts in nude mice, and this effect could be blocked by sodium cromoglycate (Cromolyn), a specific mast cell inhibitor.
Conclusions: In conclusion, our data suggest that mast cells are recruited into thyroid carcinomas and promote proliferation, survival and invasive ability of cancer cells, thereby contributing to thyroid carcinoma growth and invasiveness.

MTP 07. Molecular Mechanisms Controling Cancer Cell Growth: Role of Adipocyte-Released Factors

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Background: Epidemiologic studies provide compelling evidence that obesity is a risk factor for both cancer incidence and mortality. It is now clear that adipocytes influence several aspects of tumorigenesis. However, the molecular mechanisms involved in the adipocyte control of the malignant phenotype remain poorly understood. We have studied the mechanisms by which adipocytes may affect breast cancer cell phenotypes.
Methods: Human adipocytes and their undifferentiated precursors were incubated with serum-free medium for 8 h. Conditioned medium (CM) has been collected and added to serum-starved MCF7 breast cancer cells for different times.
Results: We have obtained evidence that CM of adipocytes was capable to elicit growth of MCF7 cells more effectively than CM of undifferentiated precursors. Similarly, CM from 3T3-L1 cells induced growth of MCF7 cells. Cytometric analysis revealed that these changes are due to reduced apoptosis instead of increased proliferation. Multiplex screening for growth factors in the CM revealed that VEGF, FGF and PDGF secretion is higher by pre-adipocytes than by adipocytes, suggesting a major involvement of these cells in promoting angiogenesis. In contrast, the content of IGF-1 produced by adipocytes is two-fold higher than that released by pre-adipocytes. Thus, IGF-1 could be a good candidate in mediating survival effect of adipocyte CM. Moreover, treatment of MCF7 with the IGF-1R inhibitor AG1024 reverted the adipocyte CM effect on cell growth.
Conclusions: In conclusion, adipocyte-derived factors promote breast cancer cell growth by inhibiting apoptosis. This effect is more evident in adipocytes than in pre-adipocytes and is, at least in part, mediated by IGF-1.

MTP 08. Bone Marrow Fibroblasts and Multiple Myeloma Progression

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Background: Fibroblasts are a key cellular component of tumour microenvironment, along with other stromal cells playing a critical role in disease initiation and progression. In this study, gene expression analysis of bone marrow fibroblasts from patients with monoclonal gammopathy of undetermined significance (MGUS, n=8) or active multiple myeloma (MM, n=10) was performed.
Methods: Differentially expressed genes were investigated through cDNA microarray (21,329 70-mer oligonucleotides; dual-label competitive hybridization), and individual gene expression patterns were confirmed by real-time RT-PCR.
Results: A number of genes functionally involved in survival (telomerase reverse transcriptase, TERT), proliferation (phospholipase A2, group IB, PLA2G1B; neuroepithelial cell transforming 1, NET1; Rho/Rac guanine nucleotide exchange factor, ARHGEF2), motility (phospholipase A2, group IB, PLA2G1B), inflammation (Interleukin 1α, IL1A; serpin peptidase inhibitor, SERPINI1) and angiogenesis (inhibitor of DNA binding 3, ID3) were found to be upregulated in fibroblasts from MM patients with respect to MGUS. In parallel, several genes were downregulated in MM fibroblasts, including the protein phosphatase PPM1L, the member of histone H2B family HIST2H2BE , the DEK oncogene, and the telomere inhibitor protein TERF1.
Conclusions: The observation of differentially expressed genes indicates an activation state of fibroblasts in MM, which very likely concur to determine a microenvironment supporting plasma cell survival and disease progression.

MTP 09. EGFR/steroid Receptors Crosstalk in Human Fibrosarcoma Cells

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Background: Recent work has shown that steroid hormones are expressed at low levels in mesenchimal cells. In HT1080 cells, the androgen promotes the migration via Rac and the androgen stimulates MMPs expression through a Src-dependent mechanism. In human fibrosarcoma, there is an increased expression of MMPs androgen-dependent that correlates with the progression and with the expression of EGFR.
Methods: Our recent work shows that EGF stimulates ER phosphorylation on tyrosine, thereby promoting the association of a complex between EGF, AR/ER, Src that activates EGF-dependent signaling pathway.
Results: In the present study, we demonstrate that in HT1080 cells E2/ER and R1881/AR trans-activate the epidermal growth factor receptor leading to the downstream signaling and to ERK activation. The metastatic progression of malignant tumors, such as fibrosarcoma, requires the association between ER /AR and EGF.
Conclusions: In vivo, a pilot study population, performed on 11 patients with soft tissue neoplasias, underlines that MMPs expression correlated with progression of anaplastic sarcoma where there is an overexpression of EGFR.

MTP 10. Role of NGAL, a NFκB-regulated gene, in the Crosstalk Between Inflammation and Cancer

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Background: The interplay between epithelial and inflammatory cells is crucial for the genesis and the establishment of carcinomas. One of the main actors in inflammatory process is NFκB which, by regulating the expression and the function of different cytokines and chemokines in inflammatory cells, stimulates the growth and its own activity in epithlial cells. Thus, NFκB establishes a network that can lead epithelial cells to undergo malignant transformation. We have demonstrated that NFκB plays an important role in thyroid cancer, especially of anaplastic type. By a differential proteomic analysis between conditioned media from parental and NFκB-null anaplastic thyroid carcinoma (ATC) cells, we identified the Neutrophil Gelatinase-Associated Lipocalin (NGAL) as a NFκB-regulated gene. The inhibition of NGAL in ATC cells determined serum deprivation-induced apoptosis and a strong reduction of their oncogenic potential, thus recapitulating some of the effects of NFκB inhibition in the same cells. Since NGAL is involved in inflammatory response and given its role in thyroid cancer, the aim of the present study is the analysis of the role of NGAL in promoting the NFκB-mediated crosstalk between inflammatory and cancer cells.
Methods: Conditioned media from parental, NFκB-null and NGAL-null ATC cells were used to induce monocytes migration through transwells.
Results: Monocytes migration was drastically reduced following exposure to conditioned medium from NFκB-null and NGAL-null ATC cells in comparison to that from parental ATC cells.
Conclusions: NGAL promoted monocytes migration in 'in vitro' assays. We are currently testing the role of NGAL in an *'in vivo'* model of inflammation-related cancer.

MTP 11. Zebrafish as a New Platform in Angiogenesis Research

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Background: Zebrafish (Danio rerio) represents a powerful model system in cancer research. Recent observations have shown the possibility to exploit zebrafish to investigate tumor angiogenesis, a pivotal step in cancer progression and target for anti-tumor therapies. Experimental models have been described in zebrafish adults, juveniles, and embryos.
Methods: We have established a novel zebrafish embryo/tumor xenograft angiogenesis assay based on the grafting of mammalian tumor cells in the proximity of the developing subintestinal vein (SIV) plexus of the embryo (Nicoli and Presta, 2007, Nicoli et al., 2007). Pro-angiogenic factors released by the tumor graft stimulate the migration and growth of sprouting SIV vessels towards the implant. The use of transgenic animals, in which endothelial cells express GFP under the control of endothelial-specific promoters, allows the observation and time-lapse recording of newly formed vessels in live embryos (Nicoli and Presta, 2007, Nicoli et al., 2007).
Results: In zebrafish embryos, antisense morpholino oligonucleotide (MO) injection induce a translational block in gene function and it can be exploited for the identification of novel gene(s) involved in tumor neovascularization. For instance, MO-induced inactivation of VE-cadherin (Nicoli et al., 2007) or of calcitonin receptor-like receptor (Nicoli et al., 2008b) orthologs result in a significant inhibition of the angiogenic process triggered by the tumor graft in zebrafish embryos.
Conclusions: Novel genetic tools and high resolution in vivo imaging techniques are becoming available in zebrafish. It is anticipated that zebrafish will represent an important tool for chemical discovery and gene targeting in tumor angiogenesis.

NOVEL BIOMARKERS FOR DISEASE DETECTION

NBD 01. Phenotypic and Molecular Study of Epithelial-Mesenchymal Transition Induced in Primary Cultures of Human Tubular Cells by High Glucose Concentration Treatment

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Background: In diabetic nephropathy hyperglycemia causes renal tubular-interstitial fibrosis by inducing tubular epithelial-mesenchymal transition (EMT) with reorganization of actin cytoskeleton and cellular adhesions. The nonreceptor tyrosine kinase Arg regulates cellular morphology and adhesion through RhoGTPase pathways. An EMT model established by hyperglycidic treatment of human tubular primary cultures has been characterized and used to study Arg involvement in EMT.
Methods: Kidney cortex, enzymatically processed, was cultured in DMEM 100mg/dl D-glucose (control cells) or in DMEM 450mg/dl D-glucose (treated cells) for 24, 96 and 168 hours. Immunofluorescence, FACS and western blot analysis with antibodies against E- and N-cadherin, cytokeratin, vimentin and α-SMA were perfomed. Stress fiber and focal adhesion density was evaluated by immunofluorescence with falloidin and anti-paxillin. Arg transcript was evaluated by Real-Time PCR and Arg protein as well as Crk and p190RhoGAP phosphorylation level by western blot.
Results: Control cells maintained a homogeneous epithelial morphology, while treated cells became fibroblast-like. In treated cells epithelial markers E-, N-cadherin and cytokeratin decreased, while mesenchymal marker vimentin increased. Stress fiber and focal adhesion density increased in treated cells. Arg transcript and protein expression decreased in treated cells as well as phosphorylation level of the RhoGTPase regulators Crk and p190RhoGAP.
Conclusions: The treatment with high glucose concentration induces EMT in tubular primary cultures with an increment of stress fiber and focal adhesion density correlated with a decreased level of Arg protein and Crk/p190RhoGAP phosphorylation. The demonstration of Arg involvement in RhoGTPase-dependent tubular EMT after hyperglycidic treatment may improve the detection of novel biomarker of diabetic nephropathy.

NBD 02. DNA Methylation and Psychiatric Disorders

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Background: Alteration of DNA methylation or chromatin configuration were found at regulatory regions of genes involved in neurotransmission and could have a key role in the genesis and/or progression of psychiatric diseases and could also be important biomarkers. In particular, REELIN, COMT, DRD2 and SOX10, demonstrated significant disease-related DNA methylation changes in post-mortem brain from schizophrenic patients. We have recently found that the BDNF gene is hypermethylated in Wernicke's area of suicide subjects compared to controls.
Methods: High resolution methylation quantitative analysis by MALDI-TOF ePyrosequencing. Real Time PCR mRNA quantitative analysis
Results: Gene-specific DNA methylation analysis of BDNF, DDO and dopamine receptor I genes in post-mortem brain samples of schizophreinic and suicide subjects demonstrated that differential DNA methylation is present in specific brain areas. qRT-PCR analysis showed that an high methylation state corresponded to low mRNA expression.
Conclusions: Our data further demonstrate that epigenetic mechanisms may underlie psichiatric conditions. These findings could have both therapeutic and diagnostic implication. We are currently investigating possible relationship between disease-related DNA methylation state in blood cells and in brain tissues in order to transfer our data to a clinical level in order to find innovative biomarkers for follow-up of the mental disease and evaluation of suicide risk.

NBD 03. Estrogen Receptor (ER) β (ERb) and ERα (ERa) May be Involved in the Pathogenesis of Testicular Varicocele.

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Background: The molecular mechanisms by which varicocele affects fertility remain undetermined. The correlation of varicocele to seminal parameters as well as sperm characteristics and function, has contradictory results. Estrogens play a key role in the male reproductive system and their function is prevalently mediated through the estrogen receptor isotypes α (ERa) and β (ERb). Human sperm express ERs, P450-aromatase and respond to estrogens. To further define the estrogen significance in male fertility and the pathophysiology of varicocele we investigated expression of ERs in sperm samples of normal and pathological subjects. In addition we investigated estradiol (E2) action on lipid and glucose metabolism, never evaluated to date.
Methods: ERs expression in sperm of normal, varicocele and oligoastenozoospermic (OAT) without varicocele patients was investigated by western blotting and transmission electron microscopy (TEM). Triglycerides content, lipase, acyl-CoA dehydrogenase and G6PDH activities were used to evaluate E2 effect on sperm metabolism.
Results: The ERs content distinguished men with normozoospermia and OAT from those with varicocele, revealing a strong reduction of ERb in varicocele samples, while ERa was almost undetectable. E2 induce energy expenditure in healthy sperm, while defective responses were observed in varicocele sperm suggesting a metabolic disorder due to the reduced ERs content.
Conclusions: Our results deepen the role of estrogens in male fertility, evidence that varicocele has a detrimental effect on sperm structure at molecular level and suggest that the ERs sperm content may be considered a molecular marker of the varicocele negative effects on spermatogenesis.

NBD 04. Isolation and Characterization of Microparticles in Sputum from Cystic Fibrosis and Primary Ciliary Dyskinesia Patients

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Background: Microparticles (MPs) are membrane vesicles released during cell activation and apoptosis. MPs have different biological effects depending on the cell from they originate. Cystic fibrosis (CF) and primary ciliary dyskinesia (PCD) are characterized by neutrophil granulocyte influx in the airways, their activation and eventually apoptosis. We investigated the presence and phenotype of MPs in the sputum, a rich non-invasive source of inflammation biomarkers, of CF and PCD patients.
Methods: Spontaneous sputum, obtained from 21 CF patients (10 acute and 11 stable) and 7 patients with PCD, were processed in order to separate MPs from the cellular component. MPs were counted and identified by immunolabelling for leukocyte (CD11a), granulocyte (CD66b), and monocyte-macrophage (CD11b) antigens and cytofluorimetry.
Results: MPs were detected by dotplot analysis and by CD66b positivity. To rule out whether bacteria or bacterial bodies could contribute to results, MPs-containing preparations were plated onto agar plates, but no bacterial growth was observed. CF sputa contained higher number of MPs in comparison with PCD sputa. Levels of CD11a+- and CD66b+-, but not CD11b+-MPs were significantly higher in CF than in PCD, without differences between acute and stable patients.
Conclusions: In summary, MPs are detectable in sputa obtained from CF and PCD patients, are not contaminated by bacteria, and are predominantly of granulocyte origin. This novel isolation method for MPs from sputum opens a new opportunity for the study of contribution of granulocytes to the lung pathology in respiratory diseases.

NBD 05. Angio-Modulation in Endothelial Cells: an Unexpected Role of Desmoglein-2 in Regulating Actin Dynamics and its Relevance to Angiogenesis Deregulation in Systemic Sclerosis

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Background: This study springs from observations on the anti-angiogenic properties of endothelial cells (EC) of Systemic sclerosis (SSc). In SSc the sufferance of microvessels leads to chronic tissue hypoxia and to capillary loss which causes vital organ failure. These features suggest an insufficient angiogenic response, despite the tissue hypoxia which characterizes the disease. Hypo-expression of Desmoglein-2 (DSG2) was shown as a major factor in impairment of angiogenesis in SSc-EC. We undertook this study to assess whether DSG2 loss of function hampered angiogenesis in normal-EC and its mechanism of action.
Methods: DSG2 was silenced in normal-EC by siRNA. The DSG2 loss of function-dependent variation of gene expression was studied by microarray and validated by RT-PCR of selected genes. Angiogenesis was studied by capillary morphogenesis in vitro and Matrigel sponge assay *in vivo*. Confocal microscopy and co-immunoprecipitation were used to show colocalization. Actin stress fibers were shown with falloidin.
Results: DSG2 loss in normal-EC was accompanied loss of angiogenesis *in vitro* and *in vivo* and by down-regulation of genes involved in EC movement, related to stress fibers assembly in the cell body, lamellipodia and filopodia. The upstream down-regulated gene product was integrin αV-β8 (ITGB8), that we have shown to co-localize with DSG2 in EC and to regulate the downstream effects of DSG2 loss. All the observed alterations were shared by SSc EC.
Conclusions: DSG2 is critical in the transduction functions of ITGB8 in N-EC and in SSc-EC. Its loss impairs the development of a correct angiogenesis program.

NBD 06. Leukocyte Telomere Length in Type 2 Diabetes as a New Diabetic Complications Marker

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Background: The key goal of diabetes management is to prevent complications. How diabetes causes long-term complications is, at present, unclear, and it is impossible to predict which diabetic patient could develop complications. In recent years, the role of leukocyte telomere length in the pathogenesis of cardiovascular disease and type 2 diabetes has been investigated. However, studies aiming to investigate the role of telomeres in the development and progression of type 2 diabetes, as well as diabetic complications, are still lacking. As a consequence, this study was aimed to verify whether leukocyte telomere length is associated with the presence of diabetic complications in a large sample of type 2 diabetic patients.
Methods: 981 subjects were enrolled, including 521 type 2 diabetic patients, of which 284 with at least one complication and 237 without complications, and 460 control subjects. Leukocyte telomere length was measured by quantitative real-time PCR.
Results: Diabetic patients with complications had significantly shorter leukocyte telomere length than diabetic patients without complications and healthy control subjects. Moreover, among diabetic patients with complications, leukocyte telomere length became significantly and gradually shorter with increasing diabetes complications.
Conclusions: The results of the study support the hypothesis that telomere attrition may be related to diabetic complications, suggesting a potential role of leukocyte telomere length measurement for complications risk assessment.

NBD 07. Plasma γ-Glutamyltransferase Fractions: Diagnostic and Prognostic Predictive Value

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Background: Serum γ-glutamyltransferase (GGT) is a biomarker of liver dysfunction, but its values are also associated with the risk of cardiovascular events related with atherosclerosis, and the onset of type 2 diabetes, irrespective of hepatic disease and alcohol consumption. Due to its low specificity GGT is not used in the risk stratification of patients. For this reason, we developed a novel high-sensitivity FPLC method, which allowed us to identify four GGT fractions (bGGT, mGGT, sGGT, fGGT, with molecular weights ranging from 2000 kDa to 70 kDa) in the human plasma.
Methods: To establish their diagnostic and prognostic value, we conducted a study entitled "GGT fractions as predictors of the metabolic syndrome and incidence of cardiovascular disease" in the context of the Framingham Heart Study (FHS, Offspring cohort 6th cycle, n = 3204), and we analysed cohorts of patients with conditions (liver disease, alcohol abuse) associated with GGT elevation.
Results: Results of these studies showed that hepatocellular damage is associated with increase of sGGT, while bGGT is connected with liver steatosis and dyslipidemia. Changes in the ratio between b- and sGGT are highly specific for identifying alcohol abuse, while f-GGT is the most abundant form in the plasma of healthy subjects.
Conclusions: In conclusion all data suggest that the GGT fraction pattern has a specific diagnostic value. These data, combined with results from FHS, will represent the basis to validate GGT fractions as specific biomarkers of hepatic, metabolic, cardiovascular disease.

NBD 08. 1H NMR-Based Metabolomic Analysis of Urine from Term and Preterm Neonates

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Background: The assessment of metabolic maturity during the perinatal period is a crucial, unresolved issue. NMR metabolomics is a technique used to non-invasively determine a snapshot of the current metabolic status of an organism by analysing intact tissue or biofluids. The aim of our study was to analyze urinary metabolic profiles in term and preterm infants in order to identify gestational age-related metabolic differences and to predict metabolic maturity at birth.
Methods: 26 healthy term infants (gestational age = 39.7 ± 0.8 wks; BW = 3292 ± 364 g) and 41 preterm infants (gestational age = 32.0 ± 3.0 wks; BW = 1830 ± 758 g) were prospectively enrolled. For each patient, a urine sample was collected non-invasively in the first 12 hours of life. The samples were frozen until analysis by 1H NMR-spectroscopy. Data from NMR spectra of urine samples were statistically analyzed by using Principal Components Analysis and Partial Least Squares-Discriminate Analysis.
Results: The multivariate statistical analysis of spectral data identified distinct metabolic patterns associated with different classes of neonates. In particular, significant differences were found between term infants and preterm infants, as well as between preterm infants of 23-32 weeks gestation and those of 33-36 weeks gestation. The individual metabolites discriminating between these experimental groups were hippurate, tryptophan, phenylalanine, malate, tyrosine, hydroxybutyrate, N-acetyl-glutamate, and proline.
Conclusions: This holistic approach appears to be a promising tool for investigating newborn metabolic maturation over time, and might lead to a tailored management of neonatal disorders.

NOVEL BIOMARKERS IN ONCOLOGY

NBO 01. Imbalance Between Cell Proliferation and Apoptosis is Associated with Epigenetic Changes in Rat Preneoplastic Liver Tissue Induced by 2-Acetylaminofluorene Exposure

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Background: The genotoxic effect of chemical carcinogens is a critical event in the initiation of tumorigenesis. However, in respect to liver carcinogenesis, it is largely unknown how the initiated cells progress to form preneoplastic foci. We investigated epigenetic changes in rat preneoplastic liver tissue associated with tumor-promoting activity of 2-acetylaminofluorene (2-AAF).
Methods: Male Sprague–Dawley rats were fed NIH-31 diet containing 0.02% of 2-AAF for 24 weeks. GSTP-positive foci were microdissected by laser capture microdissection and the methylation status of promoter CpG islands of Rassf1a, p16INK4a, Socs1, Cdh1, and Cx26 genes was analyzed by methylation specific PCR. The status of histone lysine methylation was determined by chromatin immunoprecipitation (ChIP) assay and immunohistochemistry. The extent of proliferation and apoptosis in the liver tissue was determined by double staining for GSTP/Ki-67 or GSTP/TUNEL, respectively.
Results: The results demonstrated dysregulation of the balance between cell proliferation and apoptosis. These changes were associated with altered global histone lysine methylation patterns, increased histone lysine trimethylation in the promoter regions of Rassf1a, p16INK4a, Socs1, Cdh1, and Cx26 tumor suppressor genes, and early Rassf1a and p16INK4a promoter CpG island hypermethylation in preneoplastic liver tissue of rats exposed to 2-AAF.
Conclusions: Multistage carcinogenesis following the initiation is driven primarily by carcinogen-induced epigenetic alterations. The results signify the importance of epigenetic alterations in genotoxic liver carcinogenesis and their potential role as sensitive and early biomarkers of carcinogenic exposure.

NBO 02. High Sensitivity and Specificity of Chromosomal 'Hot Spots' in Human Invasive Breast Cancer (BrCa) Associated with Circulating Nucleic Acids (CNA) Using Short Sequence Tags in Next Generation DNA Sequencing

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Background: Next generation sequencing provides high numbers of CNA serum DNA sequences that can detect trace amounts of these unique serum biomarkers in patients with cancer. Mass sequencing platforms differ significantly in the total base pairs sequenced. We have demonstrated recently the power of long sequence tags in predicting invasive ductal and lobular BrCa with the Roche 454 Titanium platform. This study now compares our long sequence tag data with short sequence tags compatible with alternate sequencing platforms such as SOLiD (ABI) or Solexa (Illumina).
Methods: DNA extracted from serum samples of 56 BCa (Stage 1 and 2) and 35 gender and age matched healthy controls (HC) was amplified using random primers and cut with N1aIII and ligated to artificial linkers harboring an EcoP15I restriction site, cut and religated. These di-tags were re-amplified, followed by a N1aIII cut and concatemization of the linker-free di-tags containing sequencing primers with identifiers. These were sequenced on a 454-Titanium system and aligned to the human genome (Build 37.1).
Results: The sequenced products consisted of up to 20 sequence tags of approximately 26 bp in length. 727,812 BCa and 531,309 (HC) sequences with a total of 13M tags were generated. An increase in hit counts of approximately 11 fold per sequence was recorded yielding 25 hot spots on 17 chromosomes where BrCa samples uniquely clustered.
Conclusions: Short sequence tags improved the sensitivity to 98 % versus 90% with long sequence tags while retaining 100 % specificity. The data provides justification for a significantly larger clinical study.

NBO 03. Analysis of the Role of Estrogen Receptor-associated Nuclear β-Actin Network in Human Breast Cancer Cell Responses to Estrogen by Gene Knock-Down and mRNA Expression Profiling

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Background: Estrogens promote breast cancer (BC) growth and progression by controlling key cellular functions via the estrogen receptor (ER) α. Upon activation, ERs translocate into the nucleus where bind to DNA at specific sites in the genome and control gene activity by recruiting transcriptional mediators. Several recent studies have demonstrated that ERα-binding sites are anchored at gene promoters through long-range chromatin interactions, suggesting that ER is involved in extensive chromatin-looping to bring genes together for coordinated transcriptional regulation. We have demonstrated that ligand-activated ERα associates in BC cell nuclei with β-actin and several actin-binding proteins, including in particular myosins and gelsolin, creating a dynamic-complex involved in transcription regulation of target genes, and suggested that ER association to a dynamic actin network might be a key determinant for chromatin-looping and, possibly, dynamic gene repositioning within nuclear domains.
Methods: To understand the impact of the protein-protein interactions described above on estrogen-dependent transcriptome in BC cells, we generated mutant MCF-7 cell clones in which gelsolin (GSNkd) or myosin-1C (MYOkd) cellular levels were knocked-down by lentiviral transduction of gene-specific shRNAs. We performed a genome-wide analysis in wild-type, MYOkd and GSNkd clones by integrating mRNA expression profiling with analysis of global ERα binding to chromatin by ChIP-Seq.
Results: Experiments performed after knock-down of the indicated genes show that gelsolin and myosin-1C are key determinants for assembly and/or stability of the investigated nuclear complex.
Conclusions: The identification of estrogen-responsive genes whose regulation is affected upon silencing of these ERα partners helps identify driver genes in breast carcinogenesis.Supported_by: UE_(CRESCENDO_contr._LSHM-CT2005-018652)_MIUR_(PRIN2008CJ4SYW_004)_Regione_Campania_(L.5/2007)_and_AIRC_(Grant_IG-8586)

NBO 04. Molecular Alterations Associated with Pulmonary Carcinoids

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Background: Pulmonary carcinoids (PCs) account for 2–5% of all primary lung tumors. The molecular alterations that contribute to PCs development are debated. Thus, we analyzed a series of sporadic PCs for somatic mutations and protein expressions of genes potentially involved in the PCs pathogenesis.
Methods: Paraffin-embedded tissues from 38 PCs (30 typical and 8 atypical) were evaluated. DHPLC and sequencing were used to analyze MEN1, TP53 (exons 5-8) and Beta-catenin (exon 3) genes. KRAS mutations at codon 12 were detected by mutant-enriched PCR after MvaI digestion. Protein expression of TP53, Menin, EGFR, β-catenin and E-cadherin were evaluated on TMAs. Statistical analysis were performed by Spearman's and Fisher's tests.
Results: In MEN1 gene we detected 1 frameshift (c.427delC), 3 missense (p.Leu89Arg; p.Ala216Thr; p.Thr541Ala), 4 synonymous (c.147T>G; c.435C>T; c.1254C>T; c.1299C) variants and an intronic mutation (c.446-5C>T) with a potential alternative splicing. Menin was predominantly expressed in the cytoplasm of PC cells. In the TP53 gene we identified 1 missense (p.255Phe) and 2 synonymous (c.387C>T, c.639A>G) variants. Two tumors, one carrying the missense, showed TP53 protein expression. The Gly12Val of KRAS gene was detected in 25% of samples. PCs showed no mutations in β-catenin gene and absence of nuclear protein expression. The cytoplasmic β-catenin expression showed a direct association with Menin ($p = 0.008$) and an inverse association with EGFR ($p = 0.048$).
Conclusions: Our data support the possible role of MEN1, TP53 and KRAS gene mutations in the development of a subset of sporadic PCs and suggest an integrated activity of Menin, β-catenin and EGFR proteins.

NBO 05. Tumor-Associated Molecular Alterations In Adipose Tissue of Patients With Colorectal Cancer

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Background: Traditionally, adipose tissue was considered to be solely an energy storage depot having only a passive function in the body. However, recent studies have shown that fat tissue exerts important endocrine functions in response to various pathological stimuli. Aim. In order to elucidate tumor-induced alterations in lipid metabolism, we tested the Fatty acid synthase (FAS) and Lipoprotein lipase (LPL) gene expression in normal and peri-tumoral adipose tissue of patients with colorectal cancer.
Methods: Fifteen consecutive colorectal cancer patients (10 males and 5 females, mean age 65.6±10.2 years) undergoing surgery of the colon were enrolled in the study. Normal and peri-tumoral adipose tissue were obtained from each of them and stored at –80°C until assayed. Levels of mRNAs for FAS and LPL were measured by reverse-transcriptase and real-time PCR. The differences in expression of two tested genes between normal and peri-tumoral adipose tissue were analysed by Mann Whitney test
Results: The levels of FAS and LPL gene expression were significantly lower in peri-tumoral adipose tissue than in the corresponding normal fat tissue (0.21±0.1 vs 0.6±0.17 and 0.37±0.12 vs 0.98±0.2, mean value ± SE, respectively, $P < 0.05$, Mann Whitney test. Data were expressed as n° molecules mRNA FAS or LPL/n° molecules mRNA β-actin.
Conclusions: Decreased FAS and LPL gene expression in the adipose tissue surrounding the tumor, presumably leads to low nutritional and energy status in that tissue, underlying malignancy-related lipoatrophy.

NBO 06. Expression of Error-Prone DNA Polymerases in B Cell Non-Hodgkin Lymphomas

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Background: Somatic hypermutation of immunoglobulin genes (SHM) is a physiological process that helps generate high affinity antibodies in germinal center B cells, and error-prone DNA polymerases are key enzymes involved in this process. Unlike polymerases that are responsible for DNA replication, error-prone DNA polymerases copy DNA with low fidelity leading to variations in immunoglobulin DNA sequences. Chronic lymphocytic leukemia (CLL) has been classified into mutated and unmutated subtypes based on the mutation status of the IGH variable region (IgVH). The latter has been associated with rapid disease progression and unfavorable outcome. Since determination of the IgVH mutations rely on direct sequencing of multiple PCR products and complex data analysis, the assay, although clinically useful, is not provided by many clinical laboratories. To determine whether error-prone DNA polymerases can be used as surrogate markers, we examined the levels of several polymerases to see whether higher levels are associated with mutations in IgVH.
Methods: Quantitative PCR was performed for the expression levels of error-prone DNA polymerases mu, eta, iota, lambda and zeta. as well as DNA editing enzymes, terminal deoxynucleotidyl transferase (TdT) and activation-induced cytidine deaminase (AID).
Results: Statistical analysis revealed no differences in the levels of mRNA expression of any of these seven enzymes between mutated and unmutated cases of CLL.
Conclusions: Our results suggest that DNA polymerases cannot be used as surrogate markers for IgVH analysis. Research is in progress to compare the expression levels of the enzymes in CLL with follicular lymphoma and diffuse large B cell lymphoma.

NBO 07. Low Levels of Soluble Form Receptor for Advanced Glycation End Products (sRAGE) in Colorectal Patients with Distant Synchronous Metastases

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Background: Receptor for Advanced Glycation End Products (RAGE) is involved in the pathogenesis of the cancer progression and metastasis. Pathological effects mediated via RAGE are pathologically inhibited by soluble RAGE (sRAGE), so that the higher sRAGE levels have been demonstrated to confer a better outcome to the patients with cancer. The aim of this study was to assess the serum levels of sRAGE in patients with colorectal cancer and to evaluate their relation to the presence of distant synchronous metastases.
Methods: Seventy-nine consecutive patients (46 males and 33 females, mean age 65.6 ±8.1 years) with histologically proven colorectal cancer were enrolled in the study. sRAGE was measured in stored fasting serum samples using an enzyme-linked immunosorbent assay (ELISA Kit, B-Bridge Int., Inc.) according to the manufactures protocol.
Results: Patients with metastases had significantly lower sRAGE compared to patients without metastases (0.50 ± 0.03 vs 0.65 ± 0.02, P=0.04 unpaired t-test, values expressed as ng/ml). This difference was also kept among male patients, whereas no differences was present in women
Conclusions: Our results show that low sRAGE levels in colorectal cancer patients are associated with the presence of metastases. Further studies are need to clearly assess whether decreased sRAGE levels in colorectal cancer patients may contribute to the progression of the disease and may be related to poor outcome.

NBO 08. Reduction of JHDM1B Levels Confers a Growth Advantage to Cancer Cells Lacking p53 Function

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Background: The histone demethylase FBXL10/JHDM1B is a nucleolar protein which preferentially binds the transcribed region of rDNA to repress the transcription of rRNA genes. Because rRNA synthesis and cell growth are tightly coupled processes, and since aberrant ribosome biogenesis is a constant feature of neoplastic progression, JHDM1B has been implicated in cell cycle regulation and tumorigenesis. However, in primary cells JHDM1B loss has been shown to reduce cell growth and to induce replicative senescence in a p15Ink4b dependent manner.
Methods: To define the contribution of JHDM1B to the biological behavior of cancer cells, we studied *in vitro* the effect of JHDM1B knock-down (KD) in breast cancer-derived cell lines.
Results: JHDM1B KD cells showed an increased ribosome biogenesis, measured as 45s precursor rRNA transcription and nucleolar size after selective silver staining. In addition, JHDM1B KD in cells retaining p53 function reduced cell proliferation and determined a higher rate of cell death and proliferative senescence. Conversely, in p53-compromised cells JHDM1B KD granted a growth advantage. In both p53 backgrounds, the growth alteration was accompanied by the transcriptional activation of p15Ink4b. These results led us to evaluate the expression of JHDM1B on a group of 65 primary breast cancers, revealing that JHDM1B expression inversely correlated with nucleolar size only in p53-mutated tumors, thereby confirming our *in vitro* observations.
Conclusions: Taken together our data indicate that epigenetic modifications of rDNA genes, through the activity of JHDM1B, are involved in the control of cell and tumor growth when p53 control function is lacking.

NBO 09. Estrogen Receptor β Recruits a Repressor Complex to ERE/Sp1 Sites of the Estrogen Receptor α Gene Promoter

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Background: Estrogens, by binding to and activating two estrogen receptors (ERα and ERβ), are critically involved in the development of the mammary gland and in the promotion and growth of breast cancer. Although ERα and ERβ have a similar structure, they act in distinct ways and have different biological functions. Recently, various studies have shown decreased expression of ERβ mRNA and protein in tumor versus normal tissues in many cancers, thus supporting the hypothesis that the loss of ERβ expression could be one of the events leading to breast cancer development.
Methods: We evaluated ERα protein and mRNA levels respectively by Western Blot and Reverse Transcription-PCR. Whereas, we used Luciferase Reporter assay and Chromatin Immunoprecipitation analysis to study the ERα gene promoter.
Results: In this study, we demonstrate that the wild-type ERβ (ERβ1), ectopically overexpressed in breast cancer cells, induces, in a ligand independent manner, down-regulation of ERα mRNA and protein content. ERα gene promoter activity was drastically inhibited by ERβ overexpression in a number of breast cancer cell lines. Moreover, Chip analyses revealed an increased binding of ERβ and ERβ/Sp1 respectively to estrogen responsive elements (ERE) and Sp1 sites on the ERα gene promoter. These events were associated with a recruitment of a corepressor complex containing NCoR and SMRT at both sites, concomitantly with displacement of RNA polymerase II.
Conclusions: Our study provides the first evidence for the molecular mechanism by which the ERβ overexpression actively represses ERα gene expression and in such way represents a good prognostic factor in breast cancer.

NBO 10. hERG1 Potassium Channels Regulate Tumor Progression in Gastrointestinal Cancers through the Modulation of the VEGF-A Pathway.

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Background: Tumour growth greatly depends on angiogenesis and cancer cells begin to promote such process early in tumorigenesis, mainly through the secretion of angiogenic factors like the vascular endothelial growth factor (VEGF)-A. We studied the relationships between hERG1, a particular type of K+ channels overxpressed in tumours, and VEGF-A expression in cancers of the gastrointestinal tract.
Methods: Such topic has been demonstrated by means of electrophysiological and molecular techniques using in vitro and in vivo models and specific anti-hERG1 drugs and siRNA.
Results: We demonstrate that hERG1 channels are functionally linked to the VEGF-A pathway in colorectal (CRC) and gastric cancer (GC) cells and they can regulate:
the secretion of VEGF-A through the transcriptional control of vegf-a gene; the HIeF-1 transcriptional activity in normoxia through the PI3K/pAKT pathway; and
the growth and angiogenesis of gastrointestinal tumour masses in different mouse models. Two main lines of evidence supported this conclusion: a) epithelial (HEK 293) cells over expressing hERG1 channels give rise to tumours of greater volume and vascularisation, compared to Mock transfected cells; b) gastrointestinal cancer cells give rise to subcutaneous tumour masses in nu/nu mice with a high degree of intratumoral angiogenesis only when they express functional hERG1 channels.
Conclusions: Hence, we have identified hERG1 as a novel molecular device which controls VEGF-A expression in cancers of the gastrointestinal tract through a hypoxia-independent regulation of HIF-1. Such results can also have a clinical significance, since we propose to use non torsadogenic hERG1 blockers in the anti-angiogenesis therapy of gastrointestinal cancers.

NBO 11. Relevance of the Retinoblastoma Protein Status in the p53-mediated Response to Chemotherapeutic Agents in Breast Cancer

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Background: The tumor suppressor protein p53 is a transcription factor that, when activated, is responsible for cell cycle arrest and apoptosis. All chemotherapeutic agents induce p53 stabilisation and activation. Therefore, a normally functioning p53 pathway in cancer cells should be highly predictive of both the response to the drugs and the clinical outcome of the patients. However, the evaluation of the p53 status - by either immuno-histochemical or molecular analysis- produced conflicting results on its prognostic/predictive value in human cancer. A possible explanation is that the pathway of the retinoblastoma protein (pRb), a major p53-downstream effector, may be altered in cancer cells, nullifying the p53 effect.

Methods: In order to gain information on the influence of the pRb-pathway status on the p53-mediated response to chemotherapy, we conducted a prospective study on the clinical outcome of 518 patients with primary breast cancers. These results were confirmed *in vitro*.
Results: Statistical analysis of disease-free survival (DFS) of patients who received standard systemic chemotherapy (5-Fluorouracil, Methotrexate and Cyclophosphamide) demonstrated that the p53 status had no predictive value if considered independently of the pRb status while in patients with a normally functioning pRb pathway (pRb neither lost nor hyper-phosphorylated), p53 resulted to be the only factor predicting the progression of the disease.
Conclusions: In conclusion, the present data demonstrated that the activity of wild-type p53 on cell proliferation after exposure to chemotherapeutic agents, can be exerted only in cancer cells with a normally functioning pRb pathway, and this must be considered when choosing therapy.

NBO 12. Expression Studies of Lysyl Oxidase and S100A4 Proteins in Normal Cortex and Renal Cell Carcinoma Primary Cultures

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Background: About 80% of sporadic clear-cell renal cell carcinomas (ccRCC) are characterized by biallelic inactivation of VHL tumor suppressor gene preventing the degradation of hypoxia-inducible factor-1a (HIF-1a). Therefore, in these ccRCCs, HIF-1 activates transcription of hypoxia-inducible genes involved in angiogenesis, cell proliferation and survival, invasion and metastasis. Among these HIF-1 target genes, the extracellular matrix enzyme Lysyl oxidase (Lox) and the calcium-binding protein S100A4 show multiple extra- and intracellular roles suggesting for each a dual function as tumor suppressor and metastasis promoter genes.
Methods: In this study we analyzed Lox and S100A4 expression, as transcripts by Real-time PCR and as intra- and extracellular proteins by western blot and immunofluorescence in 20 pairs of RCC and normal cortex primary cultures established and well-characterized from corresponding human tissue samples. Moreover, the correlation of their expression with HIF-1a level has been evaluated.
Results: Our data show a significant HIF-1a-dependent overexpression of Lox transcript in RCC cultures. Active Lox protein was more abundant in culture medium of RCC cells, instead Lox N-terminal propeptide, with putative tumor suppressor activity, was more abundant in normal cortex lysates. S100A4 transcript was significantly overexpressed in a HIF-1a-independent manner in normal cortex primary cultures and the levels of S100A4 and Lox transcripts were inversely correlated.
Conclusions: These results evidence in RCC a complex molecular network among HIF-1, Lox and S100A4 proteins involved in regulation of tumor growth and progression. Gene silencing approaches are in progress to further elucidate these results potentially useful to identify new diagnostic and therapeutic targets.

NBO 13. Novel Dyskerin-Mediated Mechanism of p53 Inactivation Through Defective mRNA Translation

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Background: In up to 60% of human cancers, p53 gene mutations are responsible for direct inactivation of the tumor suppressor function of p53. Alternative mechanisms of p53 inactivation described thus far mainly affect its posttranslational regulation. In X-linked dyskeratosis congenita (X-DC), a multisystemic syndrome characterized by increased cancer susceptibility, mutations of the DKC1 gene encoding dyskerin cause a selective defect in the translation of a subgroup of internal ribosome entry site (IRES)–containing cellular mRNAs.
Methods: In this study, we evaluated the effect of selective reduction of dyskerin levels by siRNA transfection on p53 both in human breast cancer cells and in primary mammary epithelial progenitor cells cultured as mammospheres (MS).The relationship between dyskerin expression and p53 function was also studied in a series of 119 primary breast carcinomas.
Results: siRNA-mediated reduction of dyskerin levels caused a decrease of p53 mRNA translation, protein levels, and functional activity both in breast cancer cell lines and in MS. These effects proved to be independent of the known role of dyskerin in telomerase function, and were associated with a specific impairment of translation initiation mediated by IRES elements present in p53 mRNA. In addition, in human primary breast cancers retaining wild-type p53, we found that low levels of dyskerin expression were associated with reduced expression of p53-positive target genes.
Conclusions: Our findings provide an explanation for the increased cancer incidence observed in X-DC, and suggest that a dyskerin-mediated mechanism of p53 inactivation may also occur in a subset of sporadic human tumors.

NBO 14. CD133 Expression Undergoes a Complex Post-Translational Regulation in Human Colon Cancer Cells

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Background: CD133 is a cell surface glycoprotein whose expression has been shown to characterize normal as well as cancer stem cells in several tissues but whose functions and ligands remain unknown.
Methods: In this study, the behaviour of CD133 was analyzed during colon differentiation using as a model the HT29 and CaCo2 colon cancer cells which undergo differentiation following treatment with sodium butyrate.
Results: In both cell lines, treatment induced a time- and dose-dependent decrease of CD133 protein, as assessed by flow cytometry using the AC133 antibody, but no changes in mRNA expression and gene methylation status. A reduced expression of CD133 was also confirmed by immunofluorescence using the AC133 antibody while changes were less evident when CD133 protein was evaluated by western blot using an antibody directed against the C-terminal intra-cytoplasmic region of the molecule. Different results were also obtained using the two antibodies to analyze CD133 expression in human colon cancers. It has been reported that the AC133 antibody recognizes a glycosylated epitope. We found that glycosidase treatment reduces the apparent molecular weight of the molecule but do not eliminate AC133 reactivity thus casting doubts on the proposed glycosylated nature of the recognized epitope. Moreover, we obtained preliminary evidence suggesting an intracellular localization of the protein, as confirmed by western blot analysis on sub-cellular fractions and by immunofluorescence.
Conclusions: These findings demonstrate that CD133 stem marker might undergo a complex post-translation regulation which needs to be further elucidated in order to understand its role in the definition of stem cell phenotype.

NBO 15. Characterization of New Monoclonal Antibodies Specific for the Extracellular Domain of PTPRG, a Candidate Tumour Suppressor Gene.

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Background: PTPRG gene encodes for a protein tyrosine phosphatase constituted by carbonic anhydrase-homologous, fibronectin type III domain and a cysteine-free domain in the amino terminus; a transmembrane domain and two intracellular PTPase catalytic domains. PTPRG is a broadly expressed enzyme implicated as a candidate tumour suppressor gene in breast, ovarian an lung tumors. Only one Mab raised against the extracellular domain of PTPRG expressed in prokaryotic cells was recently made commercially available but is not suitable for flow cytometric applications.
Methods: The extracellular domain of PTPRG (aa 1-736) was fused to mouse-Fc IgG3 (aa 1-237) creating a fusion protein (PTPRG-ECD-Fc), expressed and purified from HEK 293F cells. The immunization protocol was carried out by ARETA International srl (Gerenzano VA, Italy). Hybridoma cells subclones were screened on the basis of capability to recognize the native antigen by flow cytometry. Mab 6E2-B6 (IgG1) and Mab 12G5-B10 (IgM) were chosen for further analysis.
Results: The 12G5-B10 IgM antibody specifically recognized, by western blotting, the predicted 120 kDa band, the supernatant derived from Fc transfected cells being negative. This antibody does not immunoprecipitate the protein. The 6E2-B6 IgG antibody immunoprecipitate the native antigen the but do not recognize the antigen by western blotting. Both of them recognize monocytes and B cells in peripheral blood as the reference antibody Ch-PTPRG IgY.
Conclusions: The mAbs recognize native PTPRG and will be utilized, at first, to confirm the loss of PTPRG expression we described in CML (see abtract NBO 16 presented by Moratti, E. et al at this meeting).

NBO 16. Protein Tyrosine Phosphatase Receptor Type γ (PTPRG) is a Functional Tumor Suppressor Gene Specifically Down-Regulated in Chronic Myeloid Leukemia (CML).

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Background: Chronic myelogenous leukemia (CML) is the most common myeloproliferative disease. PTPRG is a receptor-type protein tyrosine phosphatase that regulates murine hemopoiesis and is expressed in myeloid cells, including CD34+ cells.
Methods: QPCR, clonogenic assays, methylation-specific PCR, flow cytometry, western blotting.
Results: Down-regulation of PTPRG increases colony formation in the PTPRG-positive megakaryocytic cell lines MEG-01 and LAMA-84, but has no effect in the PTPRG-negative cell lines K562 and KYO-1. Its over-expression has an oncosuppressive effect in all four cell lines and is associated with inhibition of BCR/ABL-dependent signaling. The

intracellular domain of PTPRG directly interacts with BCR/ABL and CRKL, but not STAT5. PTPRG is down-regulated at the mRNA and protein levels in leukocytes of CML patients in both peripheral blood and bone marrow, including CD34+ cells, and is re-expressed following molecular remission of disease. This re-expression was associated to a loss of methylation of a CpG island of PTPRG promoter that occurred in 55% of the patients analyzed. In K562 cell line, the DNA hypomethylating agent 5-aza-2'-deoxycytidine (DAC) induced PTPRG expression and caused an inhibition of colony formation that was partially reverted by antisense-mediated down-regulation of PTPRG expression.
Conclusions: These findings establish for the first time PTPRG as a tumor suppressor gene involved in the pathogenesis of CML suggesting its use as a potential diagnostic and therapeutic target.

NBO 17. Differences in miRNA Expression Profiles Between ERβ- and ERβ+ Breast Tumors in Vivo and Human Breast Cancer Cell Lines *In Vitro* Reveal a Role of these Small Non-Coding RNAs in the Hormone-Responsive Cancer Phenotype
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Background: Estrogens and their receptors are involved in physiopathology of breast cancer. While estrogen receptor (ER) α promotes cell proliferation in cancerous tissues, ERβ protects against the mitogenic effects of estrogen and, more important, appears to correlate with a better clinical outcome of the disease.
Methods: For this study we select two ERα-positive breast carcinomas groups: the first lacking (ERβ-) and the second showing (ERβ+) ERβ expression. We used Illumina Expression BeadChips to identify miRNA differentially-expressed in the two groups of tumors and, among them, to evaluate the possibility that some of them might be able to discriminate ERβ- from ERβ+ tumors. For this purpose we applied statistical analysis of microarray expression data. After normalization, the fold-change in expression was calculated to highlight miRNAs differentially-expressed between the two groups and the t-test (T-Test unpaired unequal variance-Welch) was performed to determine the significance of the differences observed.
Results: This led to the identification of 67 differentially expressed miRNAs, setting a fold-change = 1.2 and p-value <0.05 threshold, both compatible with the quality of the starting array hybridization data. Hierarchical clustering provided evidence that among these miRNA a sub-set is able to discreetly discriminate the two groups of tumors.
Conclusions: Parallel miRNA expression profiling of MCF7 cell clones lacking or expressing ERβ before and after hormonal (17β-estradiol) stimulation revealed that a significant fraction of the differentially-expressed miRNAs identified in tumor biopsies was also differentially-expressed and regulated by ERβ in this cell models, indicating cell autonomous effects of this ER subtype in breast cancer cells. Supported by: UE (CRESCENDO,contr.LSHM-CT2005-018652),MIUR(PRIN 2008CJ4SYW 004) , Regione Campania (L.5/2007) and AIRC (Grant IG-8586)

NBO 18. Loss of p27kip1 and α-Dystroglycan is a Frequent Event and is a Strong Predictor of Poor Outcome in Renal Cell Carcinoma
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[1]Università Cattolica del Sacro Cuore, Roma, Italy; [2]Ospedale Unico Versilia, Viareggio, Italy; [3]University of Modena and Reggio Emilia, Modena, Italy **Background:** Deregulation of the normal cell cycle plays an important role in malignant transformation.
Methods: In this study, expression levels of p27kip1 (p27), a negative regulator of the G1 phase, and of 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative DNA damage, were assessed by immunostaining in a series of Renal Cell Carcinomas (RCCs) and their prognostic significance was evaluated. Expression of p27 as well as of the α subunit of the dystroglycan (DG) complex, previously reported to be altered in RCC, was also evaluated by western blot analysis.
Results: Nuclear expression of p27 was reduced in a significant fraction of tumors and low p27kip1 staining correlated with higher tumor grade. Recurrence and death from clear cell RCCs were significantly more frequent in p27-low expressing tumors and Kaplan-Meier curves showed a significant separation between high vs low expressor groups for both disease-free and overall survival. Low expression of p27 as well as loss of α-DG confirmed to be independent prognostic parameters at a multivariate analysis and the simultaneous loss of both molecules defined a "high-risk" group of patients with increased risk of recurrence and death. No significant correlation with clinical or pathological parameters was found for 8-OHdG staining. Western blot analyses suggested a post-translational mechanism for the loss of alpha-DG expression and demonstrated that cytoplasmic dislocation of the protein contributes to the loss of active nuclear p27.
Conclusions: Loss of nuclear p27 is a frequent event in human RCCs and, in combination with low DG expression, could help to identify high-risk patients with clear cell RCC.

NBO 19. BARD1 Isoform Expression in Non Small Cell Lung Cancer: A Prognostic Biomarker
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Background: Lung cancer is the leading cause of cancer death worldwide, mainly due to the absence of effective screening tools for early diagnosis. We investigated whether oncogenic isoforms of BARD1, which have been correlated with poor prognosis in gynecological cancers, are also expressed in lung cancer.
Methods: We applied immunohistochemistry and RT-PCR to determine expression levels and structure of BARD1 isoforms in sections of tumor and peri-tumoral tissues from 100 non-small cell lung cancer (NSCLC) patients and in an animal model of chemically induced lung cancer.
Results: Immunohistochemistry identified reactive epitopes compatible with expression of known as well as newly identified isoforms. As in gynecological cancers, we found differentially spliced and N-terminally truncated isoforms of BARD1 expressed in NSCLC. Peri-tumoral normal tissues expressed the same pattern of isoforms, although at lower levels, while expression of any forms of BARD1 was below detection level in healthy lung tissues. BARD1 isoforms were more abundant in female than in male patient samples, and two isoforms preferentially showed gender-specific expression. One novel isoform with a partial deletion of exon four was specifically upregulated in tumor tissue. Expression of specific epitopes encoded by this isoform correlated with tumor stage and overall survival for NSCLC and with aggressive tumor stages in the animal model.
Conclusions: Our results have identified BARD1 isoforms as biomarkers for NSCLC with distinct modes of BARD1 expression in early and advanced stages of tumorigenesis. As BARD1 is the major regulator of BRCA1, structurally aberrant isoforms of BARD1 might act oncogenically and antagonistically to BRCA1 functions.

NBO 20. The K303R Estrogen Receptor α Mutation and Hormone Resistance in Breast Cancer
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Background: Aromatase inhibitors (AIs) and antiestrogens, such as tamoxifen (Tam) are frequently used for hormonal therapy of estrogen receptor (ER)-positve breast cancers in postmenopausal women. However, many patients are initially refractory or acquire resistance to either treatment. A mutation which causes a single amino acid change in the ERα hinge domain (lysine 303 to arginine, called K303R ERα), has been reported and represents a somatic, gain-of-function mutation arising in the breast resulting in a receptor which is hypersensitive to the growth effects of estrogen.
Methods: Immunoblot and anchorage indepedent assays were used to measure signlaing pathways and growth. Sequenom technology was used for genomic sequencing.
Results: We discovered that the K303R ERα mutation confers resistance to the AI anastrazole via upregulation of the IGF/PI3K/AKT signaling pathways, and blocks Tam antagonist action when engaged in crosstalk with growth factor receptor signaling pathways. The AI-resistant phenotype associated with expression of the K303R ERα mutation is dependent on activation of phospho-S305 within the receptor, and blockade of S305 phosphorylation can restore hormone sensitivity and signaling to the IGF/PI3K/AKT pathways. We are currently using mass spectroscopy to detect the mutation in breast cancer patients treated with monotherapy Tam, along with detection of specific mutations in PI3K (E545K and H1047R) and AKT (E17K), and will correlate the presence of these mutations with clinical outcomes.
Conclusions: An important consideration will be the appropriate selection of patients that may benefit from new targeted therapies to PI3K/AKT using sensitive methods for detection of these mutations.

NBO 21. Pathways of Liver Regeneration and Their Relevance to Molecular Etiologies of Liver Cancer.
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Background: Pathways involving liver regeneration initiation and termination are well understood. Mitogenic receptors of HGF and EGFR and participating non-mitogenic cytokines trigger signaling cascades leading to proliferation of hepatocytes. Extracellular matrix signaling through integrins is involved in termination of liver regeneration and control of the liver size. It was not clear whether abnormalities in any of these genes have been specifically associated with liver cancer.
Methods: Hepatocellular carcinomas of different size and etiology were subjected to copy number variant analysis by examining genomic presence of specified single nucleotide polymorphisms by oligonucleotide arrays.
Results: Most of the genes with evident high frequency of genomic deletions or amplifications were not identical to genes of agents involved in liver regeneration. Most of

the cancer related genes however were involved either in downstream signaling or as direct modulators of the effects of the signals associated with liver regeneration.
Conclusions: Liver cancer arises from multiple genomic alterations. The genes affected play meaningful roles in control of hepatocyte proliferation, by directly acting on different signaling pathways controlling cell movement, cytoskeletal organization, nuclear receptors and transcription factors. Analysis of the specific genes types provides an opportunity for better understanding of the pathways controlling proliferation of the normal hepatocytes and provides a framework for understanding the functional contributions of the genes leading to liver carcinogenesis.

PHARMACOGENOMICS AND PERSONALIZED MEDICINE

PPM 01. High BCR-ABL Expression Levels at Diagnosis May Predict Unfavorable CML Responses to Imatinib Therapy
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Background: The discovery of BCR-ABL tyrosine kinase inhibitors (TKIs) has significantly improved the history of Chronic Myeloid Leukemia (CML). However, it is still unknown if BCR-ABL transcript variants (e13a2, e14a2) predict response to IM and if high levels of BCR-ABL transcripts at diagnosis are indicative of an aggressive leukemic clone.
Methods: We assessed the BCR-ABL transcripts of 135 patients with chronic phase (CP) CML who received 400 mg daily IM. Median follow-up was 27 months. Molecular quantification of the BCR-ABL transcript was analyzed by quantitative real-time polymerase chain reaction.
Results: CP-CML patients were stratified according to BCR-ABL transcript variants and analyzed for their clinical, cytogenetic and molecular characteristics. The statistical difference between the two subgroups was the amount of BCR-ABL expression at diagnosis, with e13a2 individuals displaying high levels of BCR-ABL as compared to those with e14a2. We next clustered patients in optimal responders and suboptimal/resistant (S/R) subjects and correlated treatment response with different clinical and molecular characteristics. Only the amount of BCR-ABL transcripts at diagnosis predicted IM efficacy, with an increased number of S/R patients in the group expressing higher levels of BCR-ABL. The level of BCR-ABLIS transcript at diagnosis displayed by patients that failed TKI therapy or achieved a suboptimal response was significantly higher (106.68IS) than that of patients obtaining an optimal response (67.2IS). Increased amounts of BCR-ABL transcripts might identify CML patients at higher risk of progression.
Conclusions: Our findings suggest that high levels of BCR-ABL expression at diagnosis may identify CML patients less likely to benefit from IM therapy.

PPM 02. Cannabinoid Receptor 1 (CNR1) Polymorphisms in Human Disease.
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Background: The Endocannabinoid System (ES) is involved in a broad range of functions and in a growing number of patho-physiological conditions. In human, several studies highlighted the role of ES and of both CB1 and CB2 receptors in conditions ranging from mood disorders to neurological and psychiatric illness, altered food intake, obesity, cardiovascular, reproductive and immunological disorders. In these diseases multiple genes, environmental factors and gene–environment interactions play a crucial role, accounting for a difficult management and for an unpredictable responsiveness to therapies.
Methods: Several studies, performed by DNA sequencing and SNP analysis, suggested a role for the CB1 receptor polymorphisms in both susceptibility to develop diseases and sensibility to current treatments.
Results: To date, in the CNR1 gene at least two hot spot of polymorphic sites have been identified at the end of the codifying sequence and in the promoting region, respectively. In particular, CB1 polymorphisms have been associated with mood disorders, anorexia nervosa, schizophrenia, obesity, alcohol and drug abuse, development of addiction, increased risk for Primary Progressive Multiple Sclerosis (PPMS).
Conclusions: Here we performed a meta-analysis of preclinical and clinical data from the literature in order to dissect the true role of SNPs at the CB1 receptor in human diseases.

PPM 03. Pharmacogenetics of Transplant Medicine
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Background: Most of the immunosuppressants used in organ transplantation are characterized by narrow therapeutic index, whereby underdosing is associated with increased risk of rejection episodes and overdosing may exacerbate toxicity. Pharmacogenetics holds the potential to allow individualized dosing of immunosuppressive agents to optimize their therapeutic actions while minimizing adverse effects. Nevertheless, only thiopurine-S-methyltransferase and cytochrome P 450 3A5 genotypes appear to have sufficiently large influence to have potentialities in guiding drug dosing at the moment.
Conclusions: This may reflect the fact that available information from other polymorphisms derives almost exclusively from studies with important methodological biases. Active investigations aimed at identifying allelic variants of gene encoding for the pharmacologic targets are now ongoing. As one of the main future tasks, it is mandatory to develop mathematical models able to incorporate multiple gene polymorphisms with pharmacokinetic data and other critical information, providing algorithms able to individualize the best immunosuppressive therapy for each patient before transplantation.

REDOX REACTIONS IN HUMAN PATHOPHYSIOLOGY AND AGEING

RR 01. Exploring the Interactions Between DDB2 and Proteins Involved in DNA Nucleotide Excision Repair
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Background: Nucleotide excision repair (NER) is the principal pathway for removal of a broad spectrum of structurally unrelated lesions. In human cells, it is primarily responsible for repair of UV-induced cyclobutane pyrimidine dimers (CPD) and (6-4)-photoproducts. DNA damaged by ultraviolet light is recognized by heterodimer complex UV-DDB which comprises two subunits DDB1 (p127) and DDB2 (p48). Functional defect in UV-DDB activity has a direct relationship to decreased NER efficiency and increased susceptibility to cancer. In particular, DDB2 plays an important role in the recognition step of UV-induced DNA damage in non-transcribed regions (GG-NER), and it is mutated in Xeroderma pigmentosum (group E) patients.
Methods: In this study, we analysed the possible interaction between DDB2 and other proteins involved in NER process. We have studied the localization of DDB2 in HeLa cells transiently transfected with either control vector pEGFP-N1 or DDB2-GFP recombinant construct and then irradiated with UV-C at 10, 20, 40 and 100J/m2. The localization of DDB2-GFP was then examined 0, 5, 10, 30 min post-UV irradiation.
Results: Preliminary results showed that 5 min after irradiation (10J/m2) DDB2 increases the nuclear localization. To study the interaction between DDB2-GFP and other NER-proteins, solubilized chromatin fractions were immunoprecipitated with GFP antibody after UV-C irradiation. The co-immunoprecipitates were analysed by western blot and the results showed that the chromatin-bound fraction of DDB2-GFP is able to interact with PCNA and p21 proteins.
Conclusions: Experiments to verify and expand upon these results are in progress.

RR 02. Simple Phenols from Extravirgin Olive Oil Protects Intestinal Caco-2 Cells Against the Lipid Peroxidation Process
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Background: Complex polyphenols present in extravirgin olive oil are not directly absorbed, but undergo gastro-intestinal biotransformation, increasing the relative amount of simple phenols, tyrosol (TYR) and hydroxytyrosol (HT), entering the small and large intestine, where absorption and further metabolisation will take place.
Methods: We investigated the capacity of TYR and HT to inhibit the insult of dietary lipid hydroperoxydes on the intestinal mucosa, using cultures of Caco-2, line of human colon adenocarcinoma, that retains many of the morphological and enzymatic features typical of normal human enterocites, and studying the effect of tert-butyl hydroperoxide (TBH) treatment on specific cell membrane lipid targets, unsaturated fatty acids, cholesterol and their oxidation products, and alfa-tocopherol, extracted by Folch procedure, separated by mild saponification and quantified by HPLC.
Results: Exposure to TBH induced a significant increase of the level of MDA, the formation of fatty acid hydroperoxides and 7-ketocholesterol and the loss of α-tocopherol. Pretreatment with HT protected Caco-2 cells from oxidative damage: there was no significant detection of oxidation products and the level of the α-tocopherol was preserved. Noteworthy, TYR also exerted a protective action against fatty acids degradation. In vitro trials, where the simple phenols were tested during linoleic acid and cholesterol oxidation, gave evidence of a direct scavenging of peroxyl radicals and suggested an hydrogen atom-donating activity.
Conclusions: These findings are interesting in light of the fact that, reaching in the intestinal compartment concentration in the high uM, HT and TYR might be highly effective in preventing oxidative stress linked intestinal disorders.

RR 03. Resistance of Neuroblastoma Cell Line GI-ME-N to Redox Unbalance Involves Heme Oxygenase-1

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Background: The catabolism of the pro-oxidant heme by Heme Oxygenase-1 (HO-1), inducible form of HO, produces bioactive products, such as bilirubin, capable of exerting antioxidant, antiapoptotic and anti-inflammatory effects. Many tumours are known to express or over-express HO-1 suggesting that this enzyme might be a potential target in anticancer therapy. GI-ME-N cells, a neuroblastoma (NB) cell line without MYCN amplification, resulted particularly resistant to apoptosis after glutathione depletion by L-butionine-S,R-sulfoximine (BSO).
Methods: In our study, we have investigated the possible role of HO-1 in the resistance of GI-ME-N cells to oxidative stress and cell death after BSO treatment. In order to verify this hypothesis we treated GI-ME-N cells with a specific HO-1 inhibitor, Zinc (II) Protoporphyrin IX, or with small interfering RNA against HO-1.
Results: Our data showed that inhibition of HO-1 expression/activity, in GI-ME-N cells treated with BSO, determines decreased cell viability and increased ROS production in comparison with NB cells treated with BSO alone.
Conclusions: These results may suggest novel auxiliary anticancer chemotherapeutic approaches.Grants from PRIN 20077S9A32_002, PRIN 2008N9N9KL_002 and Genoa University.

RR 04. A Novel PDE4D Inhibitor Increases Amyloid-Beta Deposition in Neuronal Cultured Cells and Improves Memory in Rats

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Background: Amyloid-beta (A-beta) is the main constituent of amyloid plaques found in the brain of Alzheimer's disease (AD) patients and therapies aimed at reducing brain amyloidosis are expected to be effective against the neurodegenerative disorder. On the other hand, pharmacological strategies designed to enhance cerebral cAMP have been proposed as symptomatic treatments to counteract AD-related cognitive deficits.
Methods: Intracerebral microdialysis in freely-moving rats was performed to evaluate the hippocampal cAMP levels, whereas the object recognition test was used to analyse the cognitive process of memory. Quantification of A-beta secreted by neuronal cultured cells was performed with specific ELISA.
Results: Here, we present a newly synthesized selective phosphodiesterase 4D inhibitor that is able to increase hippocampal cAMP levels and improve memory in living rodents, although it stimulates secretion of A-beta in neuronal cultured cells.
Conclusions: This finding provides new insights into the therapeutic potential of PDE4 specific inhibitors and may lead to a better understanding of the physio-pathological role of A-beta.

RR 05. Hydroxytyrosol and Glucuronide Metabolites: Protective Effect in Kidney Cells

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Background: Hydroxytyrosol (HT) is an o-diphenol present in extra-virgin olive oil (EVOO), the principal fat component of the Mediterranean diet. HT is absorbed and metabolized in humans and the major metabolic pathway is the O-conjugation via glucuronidation. Since renal excretion represents its preferential disposition, it is likely that both HT and its metabolites may exert a biological effect in the renal compartment.
Methods: In this study we comparate the antioxidant activity of HT and its glucuronide metabolites (HT-3-O-glucuronide, HT-4-Oglucuronide and HT-1-O-glucuronide), investigating the capacity of the these compounds to inhibit H2O2 induced oxidative damage in kidney cells (LLC-PK1), evaluating specific parameters of the membrane lipid peroxidation process (unsatured fatty acids, cholesterol and their oxidation product)
Results: H_2O_2 treatment induced in kideny cells a significant loss of unsaturated fatty acids and cholesterol, and the formation of fatty acids hydroperoxides and 7-ketocholesterol, theirs major oxidation products. Both HT and glucuronide metabolites exerted a significant antioxidant action preserving the membrane lipids acting as scavengers of reactive radicals species.
Conclusions: HT ant its metabolites, in our experimental model, acted as scavengers of reactive radicals species; however, H_2O_2 in LLC-PK1 cells is also related to the modulation of pathways, generally associated with to oxidative stress, thus an interaction of this compound with intracellular signalling pathways activated in response to oxidative stress cannot be excluded. It is noteworthy that the protective effect of HT could be maintained also after in vivo metabolisation.

RR 06. AGEs Up-Regulates BACE1 Through Their Interaction with RAGE and NFκB activation

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Background: Although the pathogenesis of sporadic Alzheimer disease (AD) is not clearly understood, it is likely dependent on several age-related factors. Diabetes is a risk factor for AD, and multiple mechanisms connecting the two diseases have been proposed. Hyperglycemia, a consequence of diabetes, enhances the formation of advanced glycation end products (AGEs), senescent protein derivatives of glucose auto-oxidation. The interaction of AGEs with their receptor, named RAGE, elicits the formation of reactive oxygen species that are also believed to be an early event in AD pathology. Recently, different types of AGEs have been characterized, depending on the molecule from which they originate). One in particular, identified as AGE-2, is a glyceraldehyde-derived pyridinium AGE (GLAP), a toxic molecule.
Methods: To demonstrate a novel functional link between diabetes and AD, we studied the effect of two AGEs, pentosidine and GLAP, on expression and activity of BACE1, the key enzyme for amyloid β (Aβ) production both *in vivo*, in streptozotocin treated rats, and *in vitro*, in differentiated SK-N-BE neuroblastoma cells.
Results: We showed that pentosidine and GLAP were able to up-regulate BACE1 expression through their binding with RAGE and the consequent activation of NFκB. In addition, both pentosidine and GLAP were found to be increased in the brain in sporadic AD patients.
Conclusions: Our findings demonstrate that activation of the AGEs/RAGE axis, by up-regulating the key enzyme for amyloid-β production, provides a pathologic link between diabetes mellitus and AD.

RR 07. The Resveratrol-Analogue 4,4'-Dihydroxy-Stilbene Inhibits Cell Proliferation and Invasion of Human Breast Carcinoma MCF-7 Cells

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Background: The synthesis of resveratrol-analogues allowed to identify in the 4-hydroxystyryl moiety as the determinant required for their antiproliferative activity. 4,4'-dihydroxy-stilbene (DHS), a synthetic resveratrol analogue with a double 4-hydroxystyryl groups, has been demonstrated to inhibit cell proliferation of normal human fibroblasts with an higher efficiency and with a different mechanism respect to its natural parent molecule.
Methods: In this study, we have investigated the effect of the 4,4'-dihydroxy-stilbene on proliferation, adhesion and migration of MCF-7 tumour cells.
Results: Results showed an antiproliferative effect, resulting in a 25% inhibition of cell growth, as observed by cell clonogenic efficiency and cytometric analysis. In addition, 4,4'-dihydroxy-stilbene strongly reduced the MCF-7 ability of anchorage-independent growth on soft agar. Finally, a significative dose-dependent inhibitory effect on adhesion, assessed by the cell matrix assay, and migration, using a Boyden chamber, was detected in treated MCF-7 cells. In contrast, no effect was observed treating cells with resveratrol in the same experimental conditions.
Conclusions: Taken together, our results demonstrated that a) the two 4,4'-hydroxyl groups on the stilbenic backbone enhance the antiproliferative properties in cancer cells, and b) 4,4'-dihydroxy-stilbene efficiently inhibits anchorage-independent growth, migration and invasion in MCF-7 cancer cells. Therefore, 4,4'-dihydroxy-stilbene deserves further investigation in animal models to study its activity against tumour growth.

RR 08. Elastin Synthesis and Deposition are Modified During the Aging Process Depending on Fibulin 5 Availability

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Background: Loss of elasticity is a well known paradigm of aged connective tissue. Even though tropoelastin production is decreased with in-vitro aging or in cells from old donors, fibroblast from aged individuals, as a consequence of ROS-dependent increase in elastin mRNA, may be also responsible for the accumulation of the so-called elastotic material in sun-exposed skin.
Methods: In the present study we have investigated, by RT-PCR, Western blot and confocal microscopy, elastin synthesis and deposition in cultured human dermal fibroblasts isolated from young and old subjects and cultured *in vitro* (*ex-vivo* aging model) or cultured up to late passages (*in vitro* aging model).
Results: Results indicate that in both models differences in elastin mRNA expression were negligible, whereas, by Western blot, there was a significant elastin over-expression in all *in vitro* aged fibroblasts, with very few elastin aggregates on the cell surface. It has to be mentioned that tropoelastin cannot be properly organized without fibulin 5, which is known to induce elastic fiber assembly and to interact with ecSOD. In the present study, fibulin 5 was significantly down-regulated at mRNA and protein levels not only during *in vitro* aging, but also in cells from old donors.

Conclusions: In conclusion, data indicate that elastic fiber homeostasis is maintained if a correct balance between different matrix components takes place, that induction of an effective elastogenesis must be pursued by acting at multiple levels and that fibulin 5 represents a very sensitive aging marker whose down-regulation may also contribute to the altered redox balance observed with aging.

RR 09. Dopamine Peroxidation in Human Cerebellum: New Perspectives of Investigating the Pathogenesis of Parkinson's Disease.

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Background: The cerebellum is a brain area not traditionally considered dopaminergic, but it contains neuronal elements, which are indicative of dopaminergic neurotransmission. In order to investigate on the dopamine (DA) metabolism occurring in human cerebellum, we analysed this brain region by using the in gel-detection of DA peroxidizing activity, by us set out. In parallel, we assayed DA levels in cerebellar tissues in comparison with DA levels in midbrain tissue.tyrosine hydroxylase (TH) and DA transporter protein (DAT) immunoreactivity was also tested in the same tissues.
Methods: Protein mixtures from human normal cerebellum homogenates were separated in native gel and subjected to specific activity staining, by using DA and hydrogen peroxide as substrates. The reactive gel bands were analysed by mass spectrometry. DA assays were performed by High Performance Liquid-Chromatography-coupled to Electrochemical Detection (HPLC-ED). Immunocytochemical techniques were used to test TH and DAT protein expression
Results: Mass spectrometry analysis of the cerebellar reactive bands identified: Peroxiredoxins-1 (Prx-1), Prx-2, Prx-6 and DJ-1. DA levels were lower in cerebellar tissues if compared to midbrain tissue. DAT immunoreactivity was instead apparently higher than TH immunoreactivity.
Conclusions: The cerebellar peroxidative reaction revealed the presence of some proteins, peroxiredoxins and DJ-1, interplaying with the oxidative metabolism of DA. Considering that DJ-1, whose mutations are associated with autosomal recessive early-onset Parkinson's disease, has been reported to be involved in DA synthesis, as well as DAT is specific marker of DA axons, our results suggest a possible cerebellar dopaminergic system, highlighting new perspectives of investigation in the pathogenesis of Parkinson's disease.

STEM CELLS IN CANCER

SCC 01. ABCG2 is Involved in Self Renewal of Melanoma Cancer/Initiating Cells and Major Tumor Associated Antigens are Expressed by ABCG2-Positive Cells

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Background: The present model of cancer stem cells (CSC) states that tumors contain a subset of cells capable of self renewing and generating a differentiated progeny. In melanoma, initiating/stem cells have been recently identified (melanoma CSC, MCSC). In particular, our group characterized MCSC using markers CD133 and ABCG2, a member of the ABC transporter family involved in drug resistance.
Methods: We have used as *in vitro* model two melanoma cell lines, IgR39 and IgR37, obtained from the primary and from metastatic lesions of the same patient, respectively. Both of these cell lines are tumorigenic when injected into NOD-SCID mice. ABCG2+ and ABCG2- cells were sorted by cytofluorimetry and immediately injected into immunodeficient mice iv or processed for further analyses.TAA expression was studied by RT-PCR and real-time PCR.
Results: To address the first question, we have sorted ABCG2+ IgR39 and IgR37 cells, demonstrating an increase in the overall tumor mass by 3.00 and 1.75 fold, respectively, as compared to both ABCG2- and WT cells. Interestingly, ABCG2+ and WT derived tumors resulted in the same weight. Then we have analyzed the level of expression of ABCG2 in tumor xenografts. An overlap in the expression profile of the investigated TAA belonging to the CTA family and of HMW-MAA was observed at mRNA level between ABCG2+ and ABCG2- cells derived from the same parental cell line.
Conclusions: All together these results support the role of ABCG2 in self renewal in MCSC, and suggest the potential clinical effectiveness of TAA-based immunotheraputic strategies.

SCC 02. Bortezomib Specifically Targets a Subset of Hypoxia-Selected CML Stem Cells

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Background: We previously demonstrated that severe hypoxia selects LSC from CML. We found that BCR/Abl protein is suppressed in hypoxia-selected CML cells and that these cells resulted refractory to Imatinib-mesylate (IM).
Methods: Cells of the stabilized CML line K562 were used. BCR/Abl protein expression was determined by SDS-PAGE/western blotting. The maintenance of stem cell potential in hypoxic (0.3% O_2) primary cultures (LC1) was assessed by the Culture-Repopulating Ability (CRA) assay on the basis of the capacity of LC1 cells to repopulate secondary liquid cultures (LC2) incubated in normoxia.
Results: The addition of BZ at time-zero (t0) of incubation in hypoxia markedly reduced the number of viable cells, whereas that at day 1 of hypoxia was completely ineffective. In cells treated with BZ at day 1, BCR/Abl suppression was delayed at least of 1 day. The CRA assay was carried out with cells recovered from hypoxic LC1 at days 2, 3 or 7. Cells recovered at day 2 started to repopulate LC2 immediately, due to the maintenance of BCR/Abl, to peak at day 10. LC2 repopulation by cells from day-3 and from day-7 LC1 was delayed, to peak at day 21. BZ (added at t0 or at day 1) did not alter either day-2 or day-3 LC2 kinetics but significantly reduced day-7 LC2 repopulation.
Conclusions: Incubation of K562 cells in hypoxia for 1 day protects cells bulk from the toxic effects of BZ. The CRA assay defined a CML-LSC subsets hypoxia selected at day 7, BCR/Abl- typically refractory to IM but sensitive to BZ.

SCC 03. Semaphorins and Their Receptors in Lung Stem/Progenitor Cells and Lung Tumors

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Background: Several evidences link cancer with genes and pathways required for normal embryonic development, raising the possibility that cancer cells with stem cell properties, are primarily involved in tumor development. Semaphorins and their receptors, namely plexins and neuropilins, are proteins with a broader spectrum of functions, mainly involved during embryogenesis. Various semaphorins can also affect tumor progression through the promotion or inhibition of processes such as angiogenesis, metastasis and cell survival.Here we analyzed whether semaphorins and their receptors are implicated in lung stem/progenitor cells and in lung tumor development.
Methods: We isolated mouse lung stem cells, taking advantage of their ability to grow in non-adherent condition; then we performed expression assays in order to see if a panel of semaphorins and their receptors are expressed in those cells as well as in several human lung tumoral cell lines. To correlate semaphorins expression with tumor aggressiveness, we also analyzed mouse lung tumors specimens, derived from the K-RasV12 mouse model.
Results: We identified that semaphorin 3A and its receptors complex are strongly expressed in our lung stem/progenitor cells. We confirmed the presence of those proteins on the normal mouse lung tissue sections; in the tumoral context we saw an alteration of their expression during the tumor progression, from hyperplasia to adenocarcinoma.
Conclusions: We found that in stem/progenitor cells are present selective semaphorin signal pathways whose deregulation seems to be involved in lung tumor formation and progression.

SCC 04. Glucose Availability in Hypoxia Regulates the Selection of CML Progenitor Subsets with Different Resistance to Imatinib-Mesylate

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Background: Incubation under severe hypoxia of CML cell lines selects hypoxia-resistant BCR/Abl-independent Leukaemia Stem Cells (LSC) and suppresses clonogenic progenitors (LPC). Hypoxia-selected LSC exhibited "primary" resistance to imatinib-mesylate (IM), a feature highly relevant to the estabilishment of Minimal Residual Disease.
Methods: Hypoxic (0.3% O2) CML cultures established with different cell densities and glucose concentrations were treated or not with IM. BCR/Abl expression was assessed by western blotting and BCR/abl transcription by RT-PCR. Stem cell potential was determined by the Culture-Repopulating Ability (CRA) assay.
Results: The refractoriness of CML LSC/LPC to IM was explored in relation to their resistance to hypoxia or its combination with glucose shortage. It was possible to identify three hypoxia-resistant LSC/LPC subsets with different kinetic properties and IM-resistance. Glucose availability in hypoxia appeared to regulate LSC/LPC balance; In fact, hypoxia-resistant cells maintained BCR/Ablprotein expression until glucose was available. LPC surviving merely hypoxic conditions were immediately recruitable to clonal expansion upon transfer to growth-permissive secondary cultures in normoxia. Such a property, due to the prompt rescue of BCR/Abl signaling was paralleled by LPC sensitivity to IM. LSC selected under hypoxia/ischemia were instead capable of delayed clonal

expansion only and refractory to IM. IM-resistant LSC were also selected in hypoxia/ischemia from primary BCR/Abl-positive leukemia cells.
Conclusions: This study evidenced the existence of hypoxia-resistant, BCR/Ablprotein–positive LPC sensitive to IM, as well as of ischemia-selected, BCR/Ablprotein–negative LSC refractory to IM. These LSC are suitable to home in vivo within hypoxic stem cell niches and to represent the CML cell subset responsible for MRD.

SCC 05. Exploring the Function of the bcl-2 AUBP Tino by Means of its Expression Map and its Target mRNAs
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Background: The protein Tino has been first identified in our lab as a new bcl-2 AUBP endowed with destabilizing activity and found to be orthologous to the *C. elegans* protein ceMex-3. The latter plays an essential role in maintenance of cellular totipotency during worm ontogenesis and is negatively regulated by the translational repressor GLD-1, which human orthologous is Quaking protein. Lack of both ceMex-3 and GLD-1 during oogenesis leads to transdifferentiation of germ cells into neuronal and muscle cells giving rise to "worm teratomas". This prompted us to unravel possible involvement of Tino/Mex-3D in human ontogenesis and, possibly, oncogenesis.
Methods: Since Tino is transcribed but not translated, recombinant Tino or transiently expressed Tino-his and *in silico* approaches aimed to identify Tino target mRNAs have been used. A map of Tino mRNA or protein on adult and embryo mouse by in situ hybridization or immunohistochemistry analysis, respectively, has been also designed. Furthermore, reporter constructs have been used to preliminary characterize the Tino/Quaking network.
Results: Tino has been found in adult mouse testicles and some areas of embryo, such as somites and brain mesenchymal cells, which, in keeping with the role of ceMex-3 in muscle development, strongly suggests its involvement on spermatogenesis and embryogenesis. Microarray based analyses revealed that Tino could regulate a subset of mRNA, some of them involved in development and in oncogenesis.
Conclusions: First evidence of Tino translational block by Quaking let us propose a highly evolutionary conserved pathway from nematode to human.

SCC 06. Does Hepatitis B Virus X Antigen, HBx, Promote the Development of Liver Cancer Stem Cells in Hepatocarcinogenesis?
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Background: There are more than 350 million people worldwide chronically infected with hepatitis B virus (HBV) at high risk for the development of cirrhosis and hepatocellular carcinoma (HCC). HBV contributes to cancer through expression of X antigen, or HBx. Pathways implicated in the self-renewal of normal stem cells also contribute to HCC. Cancer stem cells (CSCs) consist of a subset of tumor cells that self-renew and generate tumor heterogeneity. CSCs express "stemness" genes (OCT4, NANOG, and KLF4) and "stemness"-associated genes (β-catenin and EpCAM).
Methods: Thus, experiments were designed to test whether HBx stimulates "stemness" via src mediated activation of β-catenin, the targeting of EpCAM by β-catenin released from E-cadherin and/or GSK3β complexes, and/or up-regulation of miR-181, which stimulates expression of EpCAM.
Results: HBx stimulated hepatocellular migration, growth in soft agar, and tumorigenesis of HepG2 cells in nude mice. This was associated with depressed E-cadherin through E-cadherin promoter methylation. Loss of E-cadherin was associated with an increase in β-catenin in the cytoplasm and nuclei of HBx expressing cells, and increases in β-catenin target genes (e.g., MDR1 and c-myc). HBx was also associated with up-regulation of the "stemness" genes Oct-4, Nanog, Klf-4 and c-myc and stemness-associated genes (β-catenin and EpCAM). Finally, HBx up-regulates miR-181, which is up-regulated in HCC, and promotes expression of EpCAM.
Conclusions: Hence, HBx associated up-regulated expression of "stemness" markers (Oct-4, Klf-4, β-catenin and EpCAM) and miR-181, and suppression of E-cadherin suggests that HBx contributes to HCC by promoting changes in gene expression that is characteristic of cancer stem cells.

SCC 07. Isolation and Charactriziation of Cancer Thyrospheres
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Background: Mutations affecting stem cell differentiation and may generate cancer stem cells (CSCs), which are considered the driving force maintaining indefinitely tumor-growth. It is important to evaluate the existence of CSC population also in thyroid cancer, where these cells have never been isolated and where they may sustain tumor growth, relapse after 131I treatment, and chemo-resistance.
Methods: Lan's and Fierabracci' methods were compared. Surgical normal and malignant thyroid tissue was digested by collagenase IV. Cells were seeded in serum-free stem cell medium. Fierabracci's method was adopted because it gave a higher efficiency of both normal and cancer thyrosphere formation (Lan: n=104±34/gram of tissue; Fierabracci: n=523±74/gram of tissue)
Results: Thyrospheres appeared after 7 days of cultures and displayed an irregular shape and a higher clonogenic potential after replating at single cell suspension. TaqMan Real time PCR was set up to compare the expression of stem cell markers. GAPDH was used as housekeeping gene because it displayed a similar Cycle Threshold (CT) in both normal and cancer thyrosperes. Compared to normal, the expression of Oct-4, ABCG2 and HTERT was higher in cancer thyrospheres (Oct-4: 2.6±0.7 fold).
Conclusions: These results indicate that thyroid cancers contain thyrosphere-forming cells that are less differentiated than those from normal tissue.

STEM CELLS IN TISSUE REGENERATION AND REGENERATIVE MEDICINE

SCR 01. Identification and Characterization of Cells with Stem/Progenitor Properties in Normal Kidney and Renal Cell Carcinoma
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Background: Data concerning stem cells (SCs) in kidney or in Renal Cell Carcinoma (RCC) are incomplete and the identity of renal SCs or cancer stem cells (CSCs) has not been readily forthcoming.
Methods: For the isolation of normal and cancer renal SC we used a functional approach. The single cell suspension obtained after digestion of normal kidney and RCC tissues was cultured in suspension, in a specific medium with mitogens, to form "nephrospheres". We performed a phenotypical characterization of the spheres by immunofluorescence, FACS and Real-Time PCR evaluating also some stem cell markers. To identify the SC population inside the nephrospheres we used PKH26 dye.
Results: Sphere forming efficency (SFE=n°obtained spheres/n° plated cells) was of about 0,7% for normal kidney and 0,9% for RCC. Stem cells markers appeared more expressed in the cells of the spheres if compared to differentiated cells. The cells that compose normal nephrospheres can produce tridimensional structures, likely tubules and glomeruli, in semisolid substrates, and can differentiate into epithelial and podocytic lineage with specific culture media. We can find some more fluorescent cells (PKHhigh), retaining the dye (SCs), and some less fluorescent (PKHlow/neg), replicating cells (progenitors). These populations can be sorted by FACS and only PKHhigh population can form filial nephrospheres of clonal origin.
Conclusions: The characterization of the normal PKHhigh SCs could be useful for regenerative medicine in renal pathologies. The identification of the normal SC phenotype could give some information about the CSC compartment in RCC to better understand the cellular and molecular basis of RCC.

SCR 02. Functional Proteomic Studies to Identify Oct4A Possible Interactors.
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Background: Oct4A (Octamer binding protein 4, isoform A) is a key component of the molecular circuitry which regulates stem cells self-renewal and pluripotency. Very little is known about the mechanisms through which Oct4A responds to complex extracellular stimuli and regulates stemness. In our studies we found that Oct4 was expressed both in the nucleus and the cytoplasm, implicating the presence of nuclear-cytoplasmatic translocation mechanisms. To further explore the functional network of Oct4A, in vitro and in vivo methods were used to search interacting proteins in human Dental Pulp Marrow Similar Cells (DPMSC) and NTera2, a human embryonal carcinoma (EC) stem cell line that shares many characteristics with human embryonic stem cells.
Methods: Glutathione S-transferase pull-down and co-immunoprecipitation assays identified Erk1/2 as the possible Oct4A interactor.
Results: Glutathione S-transferase pull-down and co-immunoprecipitation assays identified Erk1/2 as the possible Oct4A interactor. One mechanism by which Erk1/2 kinases ensure their specificity of action is by interacting with their substrates through docking domains, enhancing also the efficiency of phosphorylation. Oct4A sequence analysis evidenced the presence of a D-domain supporting our hypothesis. Further studies are needed to better understand the mechanism.
Conclusions: In the present study we identified Erk1/2 as a new potential interactor of Oct4A. Our results are also supported by the presence of an Erk docking domain in the Oct4A amino acid sequence, in addition to many Erk1/2 consensus phosphorylation motifs. It is thus possible that Erk1/2 phosphorylate Oct4A. This interaction could help us to better understand the functional network of Oct4A and its implications

SCR 03. Human Cardiac Mesenchimal Adult Stem Cells (hMASC) in Polymeric Scaffold: New Approach of Tissue Engineering in Cardiac Regeneration

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Background: Regenerative medicine represents a new strategy to repair tissue damage caused by myocardial infarction. To this end the realisation of bio-compatible materials, as a support for the growth of new tissue in the infarction area, could play a role of great relevance. Aim of this study is to construct natural polymeric, gradient-controlled, three-dimensional (3D) scaffolds that promote the regeneration of cardiac tissues through migration, adhesion, growth and differentiation of human cardiac mesenchymal adult stem cells (hMASC). Biomaterials play central roles in regenerative medicine and in tissue engineering as designable biochemical environments that control and guide cellular behaviour and function.

Methods: The realization of 3D collagen gels enriched with ECM proteins was performed in order to evaluate their effects on regulating hMASC adhesion, proliferation, migration/homing and differentiation. The expression of 67 kD laminin receptor (67LR) and the urokinase receptor (uPAR) as well as the production of some components of ECM were also evaluated by Western Blotting, Flow Cytometry, ELISA and Alamar Blue assays.

Results: Preliminary data showed a significant expression on hMASC of the 67LR and uPAR. Moreover, the results obtained on the proliferation, migration and production of ECM components suggest the possibility of extending the study to new natural scaffolds, provided with adjustable structure and characteristics.

Conclusions: The results obtained on hMASC suggest that the control of stem cells behaviour could have an important impact on regenerative medicine. Moreover, the biocompatible matrices could serve as templates for the growth, proliferation and differentiation of hMASC in the cellular and regenerative therapy of post-infarction cardiac tissue.

SCR 04. Generation of Induced Pluripotent Stem Cells Using Lentiviral Vectors (LVs) Encoding Reprogramming Factors (RFs) plus Cell Growth and Survival Regulatory Genes as well as Several Signal Transduction Pathway Inhibitors

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Background: Differentiated cells can be reprogrammed into induced pluripotent stem cells (iPSC) with enforced expression of multiple transcription factors.

Methods: To improve the reprogramming efficiency, we developed high titer lentiviral vectors encoding cell cycle and apoptosis regulatory genes in addition to Oct4, Sox2, Klf4, and c-Myc. The iPSC were extensively characterized by immunohistochemical staining and flow cytometry.

Results: We found that human mesenchymal stem cells and rat liver stem cells can be efficiently transduced and reprogrammed into iPSC using LVs encoding the four known transcription factors. The addition of siRNA to p53 (suppressing cell cycle) and telomerase and BclXL to prolong cell survival significantly increased the efficiency and the rate of iPSC generation. iPSC colonies were visible within a week following four RFs. Liver stem cell-derived iPSC colonies were formed within 3-4 weeks following three or two RFs. Liver stem cell-derived iPSC colonies were developed using just Oct4 alone with or without inhibitors.

Conclusions: In conclusion, the protocol for iPSC generation can be improved with high titer LVs encoding additional cellular factors regulating cell cycle progression, senescence and apoptosis as well as inhibitors. Further effort is made to delete the integrated lentiviral genomes by Cre-loxP recombination to increase the safety profile of the reprogrammed iPSC.

SCR 05. WNT'er in Liver Regenerative Medicine

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Background: Wnt/β-catenin signaling plays many relevant roles in liver pathobiology. Especially important are its roles in normal and stem cell-driven liver regeneration after surgical or chemical injury, which has been investigated in zebrafish, mice, rats and humans.

Methods: Liver regeneration and stem cell proliferation was studied in normal control (Con), β-catenin overexpressing transgenic (TG) and β-catenin conditional null mice (KO) after exposure to DDC, a known hepatotoxin. The injury and repair was characterized by serum biochemistry histology.

Results: TG mice displayed a reparative advantage in response to DDC-induced chronic liver injury. This was observed as improved serum biochemistry and increased resolution of cholestatic injury upon termination of DDC-exposure. Intriguingly, the resolution of cholestasis coincided with appearance of numerous atypical hepatocytes in the TG livers that were β-catenin+ve, A6+ve (bile duct marker) and a-fetoprotein+ve. KO, when exposed to DDC, displayed a noteworthy increase in cholestatic injury and intriguingly, the hepatocyte injury appears to resolve as evident by serum biochemistry. Histology revealed a significant atypical ductular proliferation, inflammation and fibrosis in KO exposed to DDC as compared to Con. Further evaluation revealed that KO livers at 150 days after DDC exposure were exhibiting β-catenin+ve hepatocytes. This repopulation was traced back to 30 days after DDC-exposure where 2-10 cell thick clusters of β-catenin positive hepatocytes were observed periportally. The β-catenin+ve cells have proliferative and survival advantage in KO livers and hence repopulate the entire liver within 150 days.

Conclusions: β-Catenin is playing an important role in hepatic regenerative medicine providing survival, proliferative and reparative advantage.

SCR 06. Small Hepatocyte-like Progenitor Cells in Liver Regeneration

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Background: With new models of liver injury/regeneration, we are challenged to think broadly about the nature of liver cell populations with stem-like properties, and the cellular processes that contribute to liver growth/repair/regeneration under defined pathophysiological circumstances. The small hepatocyte-like progenitor cells (SHPCs) that are observed in the retrorsine model of liver injury/regeneration provide us with one such opportunity.

Methods: We have utilized the retrorsine model of liver injury with partial hepatectomy (PH) or necrotic liver injury, alone or in combination with 2AAF or DAPM treatment, to examine cell lineage relationships.

Results: These cells are not the progeny of typical liver stem cells (oval cells) and do not conform to characteristics expected of the typical mature hepatocyte.

Conclusions: Based upon all of the evidence to date, the cells of origin of SHPCs in the retrorsine model of rat liver injury and regeneration can be described as follows: (i) distinct from oval cells (or other periportal nonparenchymal cells), (ii) reside in a parenchymal tissue niche (located in the hepatic plates, proximal to the mature hepatocytes), (iii) display a retrorsine-resistant phenotype (which may be due to lack of expression of key cytochrome P450 enzymes or other enzymes involved with intermediary metabolism), (iv) display a phenotype (and related gene expression pattern) that is not consistent with that expected for a fully mature hepatocytes. Given the unique features of these cells, we suggest that this cell population qualifies as an independent (and novel) rat liver progenitor cell type.

SIGNAL TRANSDUCTION IN ENDOCRINE AND METABOLIC DISORDERS

STE 01. Hypercholesterolemia and Risk of Alzheimer's Disease: a New Role for the Cholesterogenic Factor SREBP2.

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Background: Hypercholesterolemia has been identified as a major risk factor for Alzheimer's disease (AD). Altered neuronal membrane levels of cholesterol promotes the processing of the APP by the BACE1 to generate toxic amyloid-beta. Moreover, it has been shown the activation of cholesterogenic genes expression by SREBP2 in neuronal cells in response to accumulation of misfolded proteins, such as prions. The role of SREBP2 in hypercholesterolemia-induced activation of BACE1 has never been studied.

Methods: Male wistar rats were fed a standard diet or a high-fat diet (HF) for 20 weeks. For in vitro study SKNBE neuroblastoma cells were treated with 20 uM cholesterol for up to 48 hrs (Ch-neu). 24-OH-cholesterol, a marker of brain cholesterol synthesis, was evaluated by LC-MS. BACE1 and SREBP2 were analysed both by realtime RT-PCR and western blotting. ChIP analysis and RNA silencing were also performed to evaluate the relationship between BACE1 and SREBP2.

Results: 24-OH-cholesterol was increased in plasma and brain tissue of HF-rats, and in cytosol of Ch-Neu. Expression and activation of BACE1 and SREBP2 were increased both in HF animals brain and in Ch-neu. Moreover, Ch-cells showed SREBP2-binding on BACE1 promoter. Indeed, BACE1 up-regulation was significantly reduced, but not completely reverted, by SREBP2 siRNA in Ch-neu.

Conclusions: High-fat diet induces alterations in brain cholesterol homeostasis. Our results demonstrate that BACE1 activation not only depends on the availability of cholesterol but that neurons respond to amyloid beta deposition with a specific up-regulation of cholesterol biosynthesis, thus establishing a positive activation loop between SREBP2 and BACE1.

STE 02. Identification and Classification of Estrogen Receptor (ER)-α and Estrogen Receptor (ER)-β Chromatin Binding Regions Detected in Breast Cancer Cells by Massively Parallel Sequencing (ChIP-Seq)

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Background: In breast cancer (BC), the biological effects of ERβ are different from those of ERα. ERβ has been shown to affect ERα-positive BC cell behaviour *in vitro* and *in*

vivo. Expression studies in different ER-positive cell types suggest that ERα and ERβ share many targets genes.
Methods: To identify mechanisms by which ERβ exerts its effects on responsive genes, a global analysis of ER binding to the genome in vivo was performed by chromatin immunoprecipitation coupled to massively parallel sequencing (ChIP-Seq). MCF-7 clones expressing tagged ERβ (TAP-ERβ) underwent ChIP analysis before (-E2) or 45min after stimulation with a mitogenic dose of 17β estradiol (+E2) with ER subtype-specific Abs. The IP DNAs were subject to massively parallel sequencing with an Illumina Genome Analyzer.
Results: Comparison of tag density throughout the genome in +E2 vs –E2 samples allowed precise mapping of 6.024 binding sites for ERα and 9.706 binding sites for ERβ. Around the 40% of the binding sites were in common between the two receptor subtypes. A throughout motif search was performed and resulted in classification of all binding sites in three groups: sequences containing: (1) perfectly or imperfectly palindromic Estrogen Response Elements (ERE+; ERα: 58.89%; ERβ: 53.51%), (2) half-consensus EREs (EREhemi; ERα: 18.77%; ERβ: 18.11%) and (3) none of the above elements (EREminus; ERα: 22.34%; ERβ: 28.38%).
Conclusions: EREminus suggests indirect interactions of ERα and ERβ with the DNA and points to several TF as likely partners of these receptors in mediating their interaction with the genome via protein-protein interaction (tethering).Supported by: UE(CRESCENDO,contr.LSHM-CT2005-018652),MIUR(PRIN 2008CJ4SYW_004), Regione_Campania(L.5/2007)and_AIRC(Grant_IG-8586).

STE 03. Twist1 Plays A Pleiotropic Role in Determining the Anaplastic Thyroid Cancer Phenotype

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Background: Anaplastic thyroid carcinoma (ATC) is one of the most aggressive human tumors; it is characterized by chemoresistance, local invasion and distant metastases. While the prognosis of well-differentiated thyroid carcinoma is generally good, ATC is invariably fatal. Twist1 is a basic helix-loop-helix transcription factor that plays a key role in the initiation and progression of human cancer.
Methods: Expression of Twist1 was studied by immunohistochemistry and real time PCR in normal thyroids, well-differentiated thyroid carcinoma and ATC human samples. The function of Twist1 was studied by RNA interference in ATC cells and by ectopic expression in well-differentiated thyroid carcinoma cells.
Results: Here we show, by immunohistochemistry and real time PCR, that ATC upregulates Twist1 with respect to normal thyroid as well as to poorly- and well-differentiated thyroid carcinomas. Knockdown of Twist1 by RNA interference in ATC cells caused apoptosis and reduced cell migration and invasion. The ectopic expression of Twist1 in thyroid cells induced resistance to apoptosis and increased cell migration and invasion.
Conclusions: Twist1 plays a key role in determining malignant features of the anaplastic phenotype. Importantly, our data suggest that Twist1 could be a therapeutic target for ATC.

STE 04. Functional Proteomics of Ligand-Activated Estrogen Receptor α Interactome Reveals Hormonal Regulation of a Dynamic Nuclear Actin Network in Breast Cancer Cells

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Background: Estrogen regulation of target cells proliferation results from promotion of both cell growth and survival. These effects are mediated by an interplay of genomic and extra-genomic pathways that, in hormone-responsive breast cancer cells, determine cell cycle progression inducing the transcription of responsive genes such as cyclins. Estrogen receptor α (ERα) is a key mediator of these mitogenic actions, behaving as transcription factor that, once activated by the hormone, undergoes structural changes resulting in its nuclear translocation and docking to specific chromatin sites. In the nucleus ERα assembles in multiprotein complexes determining finally the effects of the genomic estrogen signalling. Understanding the effects of ligand-activated ERα in target cells requires identification of the molecular partners acting in concert with this nuclear receptor to transduce the hormonal signal.
Methods: Functional proteomics was applied to identify proteins interacting with ligand-activated ERα in MCF-7 cell nuclei. Cell clones expressing human receptor fused to a Tandem Affinity Purification-tag were used to purify native nuclear ER-containing complexes.
Results: This led to the identification of several proteins, some of which had not been previously found associated to ERα. The complex comprising β-actin, myosins and proteins involved in actin filament organization and dynamics was first investigated.
Conclusions: Time course and co-immunoprecipitation analyses indicated that complexes containing ERα and β-actin are assembled in the nucleus after receptor activation. The involvement of such complexes in the regulation of gene transcription is finally suggested by the co-recruitment of both ERα and β-actin to the promoter of estrogen-responsive genes. Supported_by_UE_(CRESCENDO_contr._LSHM-CT2005-018652)_MIUR_(PRIN 2008CJ4SYW_004) Regione_Campania (L.5/2007)_and_AIRC_(Grant_IG-8586).

STE 05. Analysis of the Nuclear Estrogen Receptor α Receptosome of Human Breast Cancer Cells

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Background: ERα is involved in mammalian gland epithelial cells growth regulation and plays a role in mammary carcinogenesis. ERα acts mainly in nucleus as a transcription factor, regulating target genes activity directly by binding to chromatin. This activity is modulated by protein components interacting with ERα, which are mostly still unknown. Identifying ER interactors gives a better understanding of molecular mechanisms mediating hormonal effects on breast cancer (BC) development and reveals potential new therapeutic targets against hormone-responsive BC.
Methods: In order to identify novel ERα-containing nuclear protein complexes, Tandem-Affinity-Purification (TAP) was applied to isolate native ER-containing protein complexes from hormone-stimulated human BC cells. To this end, two recombinant ERα were generated by fusing a TAP-tag to either N- or C-terminus of the protein. N-TAP-ERα and C-TAP-ERα fusion proteins obtained, that were both functional, as determined by various 'in vivo' assays, were stably expressed in MCF-7 cells under control of tet-inducible promoters. Receptor-containing protein complexes extracted from estrogen (E2)-stimulated cell nuclei were purified by sequential affinity chromatography and glycerol gradient centrifugation and analyzed by mass spectrometry (nanoLC-MS/MS).
Results: This led to identification of about 300 receptor-interacting proteins with C-TAP-ERα and a similar number of N-TAP-ERα interactors.
Conclusions: Several complexes were identified with only one of two fusion proteins screened, suggesting specific protein interactions at the transcriptional activation functions TAF-1 and TAF-2 of the receptor molecule. The vast majority of these proteins represents novel receptor partners whose activity reveals an involvement of agonist-activated ERα in multiple steps of gene transcription and mRNA maturation cascade. Supported_by:UE(CRESCENDO,contr.LSHM-CT2005-018652),MIUR(PRIN2008CJ4SYW_004),Regione_Campania(L.5/2007).

STE 06. Hypoxia and Angiogenesis During the Progression of Human and Murine Non-Alcoholic Fatty Liver Disease

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Background: Profibrogenic mechanisms operating in non-alcoholic steatohepatitis (NASH) progression are still to be fully elucidated. In particular, there is a lack of data on the role of hypoxia and the related angiogenic response in the pathogenesis of this disease. In this study we investigated whether hypoxia, through hypoxia-inducible factors (HIF), may affect angiogenic response of hepatic parenchymal and non-parenchymal cells.
Methods: Morphological analysis (immunohistochemistry or IHC; indirect-immunofluorescence or IIF) was performed on liver specimens from C57BL/6 mice, receiving methionine-choline deficient (MCD, 4 weeks) diet and from patients with NAFLD/NASH. Signal transduction in HepG2 cells was evaluated by integrating cell, molecular biology and morphological techniques.
Results: IHC performed on liver specimens from both MCD mice and human adult patients revealed a positive hepatocyte staining for VEGF in periportal and centrilobular areas, although colocalization of VEGF with HIF-2α was detected mainly in steatosic hepatocytes around the centrilobular area. In human specimens, HIF-2α also co-localizes with SERPINB3, a serine protease inhibitor recently shown to be hypoxia-dependent and able to mediate migration of human myofibroblasts. Finally, by using a oleic-acid dependent model of fat-laden HepG2 cells (fl-HepG2)we found that exposure of fl-HepG2 to hypoxia resulted in a significant increase of HIF-1α, VEGF, VEGFR2 and HIF-2α expression, particularly evident after 48hrs.
Conclusions: These preliminary results indicate that hypoxia and hypoxia-related VEGF expression, events possibly affecting fibrogenesis and angiogenesis, seem to develop during natural history of NAFLD progression towards NASH in both human patients and MCD mice.

STE 07. Environmental and Epigenetic Regulation of the Diabetes-Related PED/PEA-15 Gene

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Background: The molecular details of gene–environment interplay in type 2 diabetes (T2D) onset have recently received great attention. PED/PEA-15 is a gene commonly overexpressed in tissues from T2D individuals and healthy subjects at high risk of developing diabetes (such as first degree relatives). Indeed, overexpression of the ped/pea-15 gene in mice, impairs glucose tolerance and leads to diabetes in conjunction with high-fat diet treatment. We have evaluated the influence of reactive oxygen species (ROS) on PED/PEA-15 regulation, representing oxidative stress a mechanism by which aberrant epigenetic programming leads to T2D.
Methods: Chromatin Immunoprecipitation Assays have been performed to identify histone marks and histone-associated proteins.
Results: Incubation of THP-1 cells with hydrogen peroxide determines an increase in the binding of the transcription factor nuclear factor kB (NF-kB) to its response element on PED/PEA-15 promoter causing an up-regulation of PED/PEA-15 gene. This effect was accompanied by an increase both in dimethylation of lysine 4 (H3K4me2) and acetylation of Lysine 9 and Lysine 14 (AcH3) at PED/PEA-15 promoter and coding region, indicating that ROS determine chromatin modifications on PED/PEA-15 gene. Further co-immunoprecipitations experiments performed using NF-kB-p65 followed by H3K4me2 and AcH3 antibodies revealed both H3K4me2/NF-kB and AcH3/NF-kB complexes at the PED/PEA-15 promoter indicating that NF-kB recruitment is accompanied by histone di-methylation and acetylation at PED/PEA-15 promoter.
Conclusions: These results suggest that an inflammatory stimulus like hydrogen peroxide alter the epigenetic regulation of the PED/PEA-15 gene determining an increase of its expression levels.

STE 08. Beneficial Effects of St. John's Wort and Hyperforin Against Cytokine-Induced Beta-Cell Pro-Inflammatory Response, Dysfunction and Death in Rat and Human Pancreatic Islets.

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Background: Inflammatory cytokines produced in response to autoimmune processes or metabolic stimuli contribute to the deficiency in b-cell mass and function occurring in both type 1 and type 2 diabetes. The extract of Hypericum perforatum (St. John's wort, SJW), an herbaceous plant used in phytotherapy as anti-depressant, and its component hyperforin (HPF) were previously shown to inhibit cytokine-induced STAT-1 and NFκB activation in INS-1E b-cell line. Objective of this study was to assess the protective effects of SJW and HPF on cytokine-exposed rat and human pancreatic islets.
Methods: Isolated rat or human islets, incubated with cytokines and/or SJW extract (50-200 mg/ml) or HPF (1-3 mM), were used for functional, ultrastructural and RT-PCR gene expression studies and for simultaneous evaluation of apoptosis and necrosis by ELISA technique. STAT-1 and NFκB activation was assessed by electrophoretic-mobility-shift-assay.
Results: SJW extract and HPF corrected cytokine-induced dysfunction in rat and human islets and inhibited STAT-1 and NFκB activation as well as mRNA expression of pro-inflammatory genes, such as iNOS, COX-2 and CXCL10. Furthermore, the increase in apoptosis and necrosis found upon 48-h exposure of islets to cytokines was fully prevented by SJW and partially by HPF. At ultrastructural level, the vegetal compounds also avoided mitochondrial alterations and loss of insulin granules. Quantitative analysis confirmed a significant reduction of apoptosis by SJW.
Conclusions: SJW extract and hyperforin target key mechanisms of cytokine-induced beta-cell injury, thereby improving beta-cell function and survival. Thus, they represent a promising approach for prevention or limitation of beta-cell loss in diabetes.

STE 09. Identification of Estrogen Receptor β Interacting Proteins in Breast Cancer Cell Nuclei by Tandem Affinity Purification and NanoLC-MS/MS

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Background: Estrogens exert their control on genome activity by binding and activating the two nuclear Estrogen Receptors (ER) -α and -β, members of the steroid/nuclear receptor superfamily of transcriptional regulators. ER ligands induce a change in receptor conformation resulting in nuclear translocation, protein dimerization and binding to chromatin, where they cooperate with distinct multiprotein coregulator complexes to control gene transcription. In breast cancer cells this leads to the causal relationships between estrogen actions and the hormone-responsive tumor phenotype. Although ERs share a conserved modular structure, they show strikingly different functional roles in onset and progression of hormone-responsive breast cancer (BC). In particular, ERβ exerts a modulatory activity on ERα-mediated estrogen signalling and stimulation of cell proliferation by mechanisms still not fully understood.
Methods: Identification of functional protein-protein interactions involving ERβ is an useful approach to investigate the role of this receptor subtype in estrogen signalling and to identify different signal transduction pathways discriminating from ERα-depending cascades. To this aim we applied a proteomics work flow, after stably expressing human ERβ fused to a TAP-tag in estrogen-responsive MCF-7 cells, based on Tandem Affinity Purification (TAP) and nanoLC-MS/MS to identify ERβ interactome in this cell type.
Results: This led to the identification of the 303 proteins that co-purify with ERβ from nuclear extracts.
Conclusions: Functional analysis brought to light several new molecular partners of this receptor subtype that represent nodal points of a large protein network controlling multiple biological processes and functions in BC cells. Supported by: UE (CRESCENDO_contr_LSHM-CT2005-018652), MIUR (PRIN_2008CJ4SYW_004), Regione Campania_(L.5/2007) and AIRC (Grant_IG-8586).

STE 10. CLA Modifies Protein Expression Profile in Rat Hepatocytes

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Background: It has been recently proven that conjugated linoleic acid (CLA) reduces body fat mass and has antitumor activity in breast cancer. On this basis we decided to investigate the ability of CLA to modulate the proteomic profile in liver.
Methods: We asses the modification of protein expression in rat hepatocytes incubated with CLA; the proteomic profile was analyzed using two dimensional electrophoresis. Protein spots were identified by mass spectrometry.
Results: In CLA-treated hepatocytes two proteins showed a reduced expression: the estrogen sulfotransferase, isoform 3 (ST1E3) and tha isoamyl acetate-hydrolyzing esterase 1 homolog (IAH1).
Conclusions: The reduction of body fat mass induced by CLA causes liver enlargement due to an increased lipid content. The lipogenetic activity seems to be due to the reduction in fat mass, and to the consequent metabolization of blood glucose to fatty acid in the liver rather than in the adipose tissue. Here we used an "*in vitro*" model and this connection between fat tissues and liver cannot be evaluated. Nevertheless, we demonstrated that also a direct effect of CLA on lipid accumulation in liver exists, demonstrated by the reduced expression of the IAH1, involved in lipid degradation. It is also known that CLA has an antitumor activity, even though the mechanism of action is not clear: some studies have suggested an involvement of the estrogen signalling pathway. We demonstrated a reduced expression of an estrogen sulfotransferase, the ST1E3, which is involved in the inactivation of free estrogens to inactive solfoconjugates. (This work is supported by MIUR-PRIN 2008)

STE 11. Identification of Novel Kinases Involved in Thyroid Carcinogenesis

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Background: Thyroid cancer is the most frequently occurring malignancy of the endocrine system. Significant advances in understanding the molecular basis of thyroid carcinogenesis and identifying tumour-initiating genetic have been made. The cure probability depends on histotypes, tumour stage and sensitivity to radioactive iodine. Therefore, resistant thyroid carcinomas represent a very attractive target for biological therapies. Many kinases are estimated to function as cancer genes, as tumor cells depend on their activities to growth and proliferate.
Methods: We have performed a loss-of-function genetic screen by using a library of synthetic small interfering RNA (siRNA) targeting the entire human kinome to identify kinases whose knock-down is affecting thyroid cancer cell growth. In our screening we have targeted a total of 646 genes, including all known kinases and kinase-associated genes, and we have identified a set of about 100 human kinases, able to reduce cell viability.
Results: We have then performed a secondary validation screening with an independent set of siRNAs targeting a distinct mRNA region of the highest ranked 36 kinases. This led to the identification of 21 kinases important for cell viability of different thyroid cancer cell lines. They include logical candidates, like cell cycle kinases (CDK4, HIPK1) and signaling kinases (FYN, AKT2), together with some new interesting class of genes, like the ephrines (EPHs) family and the JNK-p38 related family.
Conclusions: Understanding the role of these new kinases in the biology of thyroid cancer cells and testing their inhibition alone or in combination will open new possibilities to translate genetic information into clinical practice.

STE 12. Identification of a Gene Network Controlled by Estrogen Receptor α in Human Breast Cancer Cells by MiRNA Expression Profiling and ChIP-Seq

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Background: Estrogens control several biological processes by acting as ligands of estrogen receptor α (ERα), member of nuclear receptor super-family of transcriptional regulators. Following estrogen activation, ERα translocates to the nucleus where regulates the transcription of target genes, binding specific estrogen response elements (EREs) on DNA. MicroRNAs (miRNAs) are 18-25nt noncoding segments of RNA that negatively regulate gene expression at post-transcriptional level by partially base pairing with target gene mRNA sequence.
Methods: In order to study the effects of estrogens on miRNAs expression profiles we performed a time course analysis using human breast cancer MCF-7 and ZR-75.1 cells. RNA extraction was performed from the above mentioned cell lines before and after different times of stimulation with 17β-estradiol (E2). The RNA was hybridized on Illumina Human V2 miRNA BeadChip that measure the levels of 1.145 miRNAs.
Results: This led to the identification, in both cell lines, of a subset of miRNAs that display the same response to E2 and cluster in two concordant groups, each characterized by defined kinetics of miRNA accumulation or decrease. To identify the mechanisms by which ERα exerts its effects on estrogen-responsive genes, the estrogen-dependent miRNA expression profiles were integrated with global in vivo ERα binding sites mapping in the genome by ChIP-Seq. In addition, data from miRNAs and mRNA expression profiles obtained under identical experimental conditions were aligned to identify relevant miRNA target transcripts.
Conclusions: The results suggest that miRNAs modulated by ERα represent a novel genetic pathway to impact estrogen-dependent developmental processes and hormone-related pathologies. Supported_by: UE(CRESCENDO_contr._LSHM-CT2005-018652),_MIUR_(PRIN_2008CJ4SYW_004), Regione_Campania (L.5/2007)_and_AIRC_(Grant IG-8586).

STE 13. In Lysinuric Protein Intolerance Monocytes Exhibit a Defective System y+L Activity

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Background: Lysinuric Protein Intolerance (LPI, MIM 222700) is an autosomic recessive aminoaciduria caused by defective cationic amino acids (L-arginine, L-lysine, L-ornithine) transport at the basolateral membrane of epithelial cells of intestine and kidney. The mutated gene in LPI is SLC7A7, coding for the light subunit y+LAT1 of the transport system y+L. Clinically LPI is characterized by hyperammonemia, gastrointestinal symptoms, failure to thrive, renal disease, osteoporosis, hematopoietic abnormalities, and pulmonary manifestations that range from subclinical interstitial lung disease to a severe fatal pulmonary alveolar proteinosis (PAP). In PAP alveolar spaces of the lungs are excessively filled with lipoproteinaceous material because alveolar macrophages are defective in surfactant clearance. In this study we investigated the molecular mechanisms underlying LPI-associated PAP.
Methods: Monocytes and fibroblasts were isolated from a patient affected by LPI or from healthy donors. System y+L activity was determined through 3H-arginine uptake. The expression of system y+L transporters was evaluated by qRT-PCR determining the expression of SLC7A7/y+LAT1 and SLC7A6/y+LAT2.
Results: In both normal and LPI monocytes, the expression of SLC7A7 is very high while in fibroblasts is extremely low,. On the contrary, SLC7A6 is high in fibroblasts and very low in monocytes. Because of the mutated y+LAT1 protein, system y+L activity in LPI monocytes is not detectable; LPI fibroblasts are, conversely, endowed with a functional system y+L thanks to the presence of y+LAT2 that compensate the defective y+LAT1.
Conclusions: Monocytes/macrophages, displaying fully the LPI defect, play a key role in the pathogenesis of the pulmonary manifestations.

STE 14. Regulation of Arginine-Nitric Oxide (NO) Metabolic Pathway by PKC in Human Endothelial Cells: Arginase Activation and eNOS inhibition

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Background: Arginine availability appears of peculiar relevance in the vasculature, where it contributes to the regulation of vascular tone. In endothelial cells, arginine exerts its biological functions as substrate of two alternative pathways: endothelial nitric oxide synthase (eNOS), which catalyzes the production of nitric oxide (NO), the most important vasodilator in vivo, and arginase, which hydrolyzes arginine to ornithine and urea. A reduced production of NO in the vessel wall is likely the key initiator of endothelial injury underlying several pathological conditions, as atherosclerosis, coronary artery disease and diabetic vasculopathies; however, the reasons for such decrease are not understood. The present study aims to characterize arginine-NO metabolic pathway in human aortic endothelial cells, with particular attention to the role of PKC, known to modulate eNOS phosphorylation.
Methods: Intracellular arginine content was measured by HPLC. Arginase and eNOS expression were evaluated by qRT-PCR and Western Blot, whereas enzymatic activities were estimated through conversion of 14Carginine to urea or citrulline, respectively. NO production was determined with a fluorimetric approach.
Results: The activation of PKC associates with a transient increase of intracellular arginine content and a marked stimulation of arginase expression and activity. In parallel, the changes observed in the phosphorylation pattern of eNOS result in the inhibition of its activity and in the decrease of NO production.
Conclusions: The activation of PKC in human endothelial cells shifts the metabolism of arginine from NO synthesis to arginase-dependent production of ornithine and urea. This metabolic deviation may contribute to the endothelial dysfunction associated with conditions of PKC overactivity.

STE 15. The Mammalian Life-span Determinant p66shcA Mediates Obesity-induced Insulin Resistance

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Background: Obesity and metabolic syndrome are mechanistically linked to type II diabetes and accelerated body ageing, yet the underlying molecular mechanisms are incompletely understood. Mice lacking the p66 kD isoform of the Shc adapter molecule (p66KO) live longer and are leaner than wild type animals, suggesting that this molecule may have a role in metabolic derangement and premature senescence by overnutrition.
Methods: To address the role of p66shc in obesity-associated diabetes, we have crossed p66 mice with Ob/Ob (leptin-deficient) mice, an established genetic model for overnutrition, excess body weight and insulin resistance.
Results: We found that p66 deficiency exerts a small protective effect on fat accumulation and premature death in lepOb/Ob mice; more strikingly, p66 inactivation improved glucose tolerance in these animals, whitout affecting (hyper)insulinaemia and independent of body weight. Protection from insulin resistance was cell-autonomous, since isolated p66KO preadipocytes were relatively resistant to insulin desensitization by free fatty acids *in vitro*. Biochemical studies revealed that p66shc promotes the signal-inhibitory phosphorylation of the major Insulin transducer IRS-1, by bridging IRS-1 and the mTOR effector p70S6 Kinase, a molecule previously linked to obesity-induced insulin resistance. Importantly, IRS-1 was strongly up-regulated in the adipose tissue of p66KO lepOb/Ob mice, confirming that effects of p66 on tissue responsiveness to insulin are largely mediated by this molecule.
Conclusions: Taken together these findings identify p66shc as a major mediator of insulin resistance by excess nutrients, and by extension as a potential molecular target against the spreading epidemic of obesity and type II diabetes.

STE 16. Evaluation of Epicardial Adipose Tissue (EAT) in Metabolic Syndrome Patients

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Background: Epicardial and myocardial fats increase with degree of visceral adiposity and possibly contribute to obesity-associated cardiac changes. Echocardiographic epicardial fat thickness is a new and independent marker of visceral adiposity. The aim of this study was to test whether echocardiographic epicardial fat is related to myocardial fat.
Methods: Twenty consecutive Caucasian men (body mass index 30.5 +/- 2 kg/m(2), 42 +/- 7 years of age) underwent transthoracic echocardiography for epicardial fat thickness, morphologic and diastolic parameter measurements, hydrogen-1 magnetic resonance spectroscopy for myocardial fat quantification, and magnetic resonance imaging for epicardial fat volume estimation. Hydrogen-1 magnetic resonance spectroscopic myocardial fat content, magnetic resonance imaging of epicardial fat volume, and echocardiographic epicardial fat thickness range varied from 0.5% to 31%, 4.5 to 43 ml, and 3 to 15 mm, respectively.
Results: Myocardial fat content showed a statistically significant correlation with echocardiographic epicardial fat thickness ($r = 0.79$, $p < 0.01$), waist circumference ($r = 0.64$, $p < 0.01$), low-density lipoprotein cholesterol ($r = 0.54$, $p < 0.01$), plasma adiponectin levels ($r = -0.49$, $p < 0.01$), and isovolumic relaxation time ($r = 0.59$, $p < 0.01$). However, multivariate linear regression analysis showed epicardial fat thickness as the most significant independent correlate of myocardial fat ($p < 0.001$).
Conclusions: Although this study is purely correlative and no causative conclusions can be drawn, it can be postulated that increased echocardiographic epicardial fat accumulation could reflect myocardial fat in subjects with a wide range of adiposity.

TUMOR IMMUNITY

TIM 01. The Role of γ δ T cells Infiltrating Colorectal Cancer

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Background: The role of the adaptive immune response in controlling the growth and recurrence of human tumors has been controversial. Several papers have demonstrated that *in situ* analysis of tumor-infiltrating immune cells may be a valuable prognostic tool in the treatment of colorectal cancer.

Methods: Because of the antitumoral activity of γ δ T cells, we characterized the cancer-infiltrating γ δ T cells in a cohort of 70 patients in terms of phenotype and effector functions.

Results: Our results showed, that V δ 1 T cells were the predominant population in the vast majority of specimens and upon short term in vitro stimulation produced IL-10. Vγ9Vδ2 T cells were found uniformly at lower proportion in most of the patients, but dominated the γ δ T cell response in 9 out of 70 patients. Tumor-infiltrating Vγ9Vδ2 T cells had a predominant terminally-differentiated effector memory (TEMRA) CD45RA+CD27- phenotype, expressed cytotoxic molecules as perforin and granzyme B and upon short term in vitro stimulation with BrHPP these cells produced pro-inflammatory cytokines as IL-17 and IFN-γ in different combinations. Localization of Vγ9Vδ2 T cells to cancer was not due to a biased predominance of this subset in cancer patients because Vγ9Vδ2 T cells obtained from the peripheral blood of the same patients had a naïve, central memory and effector memory phenotype, while terminally-differentiated effector memory cells were very poorly represented.

Conclusions: Our results highlight the role of Vγ9Vδ2 T cells against colorectal cancer cells and might provide a strong rationale for the use of pharmacological drugs, as T cell agonists, in immunotherapies.

TIM 02. Use of Antibody-Coated Beads for Expansion of CD8+ T Cell Subsets for Immunotherapy

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Background: A subset of the antigen–reactive CD8+ T cells develop into memory cells when encountering a particular antigen for the first time. With repeat encounters of the same antigen, the antigen-reactive CD8+ T cells acquire a phenotype in which they express increased levels of a number of NK cell markers as well as constitutively express high levels of cytolytic effector molecules such as granzyme A, granzyme B and perforin. Expression of CD8 on NK-like CD8+ T-cell distinguishes it from the CD4 expression on NKT cells. While these cells are able to mediate cytolytic function, they exhibit poor proliferative responses to polyclonal T cell stimuli and thus have been labeled as terminally differentiated or replicative senescent cells. The numbers of these terminally differentiated cells are often expanded in older individuals or in tumor bearing individuals and potentially could be used as a good source of effector cells for direct in-vivo cancer immunotherapy.

Methods: The use of these NK-like CD8+ cells for in-vivo cancer immunotherapy will be enhanced by the development of methods that bridge these cells with tumor cells and the identification of reagents that are able to induce the activation and proliferation of these NK-like CD8+ cells. This series of studies explored the possibility of using bead bound antigen as one approach to induce activation and proliferation of this subset.

Results: The results indicated that bead-bound antigen is a useful tool to inducing responses by the NK-like CD8+ cells.

Conclusions: The application of these techniques to immunotherapy will be explored further.

TIM 03. Mast Cell at Crossroads Between Tolerance and Control of Adaptive Immune Response

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Background: CD4+CD25+ regulatory T (Treg) cells are known as modulators in MCs response and homeostasis. We are investigating the role of OX40:OX40L axis, a receptor:ligand pair known to modulate Treg cell immunosuppressive activity. Interaction between OX40L on MCs and OX40 on Tregs elicited an inhibitory effect on MC degranulation, but not on IL-6 and TNF-α production through the increase of cAMP levels. Since these biological events follow the aggregation of the Fc€RI receptor, we investigated the molecular pathways following MC-Treg interaction.

Results: We found that the Fyn, Gab2 and Akt phosphorylation in IgE/DNP activated MCs was decreased in presence of Tregs cells but not when MCs were co-cultivated with Treg OX40-/- cells. Moreover, MCs activated in the presence of Treg cells showed reduced Ca2+ influx, independently of PLC-?2 or Ca2+ release from intracellular stores, but is dependent on arrest of STIM1 traslocation, a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. By immunofluorescence, EM localization, and surface biotinylation we demonstrate that STIM1 migration from ER-like sites to the plasma membrane upon depletion of the Ca2+ store is blocked in IgE/DNP activated MCs co-cultivated with Treg cells.

Conclusions: The influence of MCs/Treg interaction in presence of Teff cells is regulated by MCs cytokine milieu that skew both Tregs and Teff cells into IL-17 producing T cells (Th17). All this data suggest novel molecular pathways that can be used as target of therapeutic approaches to the therapy of autoimmune.

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