



# SIP Meeting

**“Beyond the bench...preparing  
your career transition”**

*Stazione Zoologica, Naples  
2-3 December 2009*

Programme & Abstracts



**D B P C M**  
Dipartimento di Biologia e Patologia Cellulare e Molecolare  
"Luigi Cattaneo"

**Organizing Secretariat**



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# PROGRAMME

**2th December, 2009**

**15.00-15.30 Welcome Addresses**

*Chairs: Giancarlo Vecchio, Silvestro Formisano and Roberto Di Lauro  
(Naples)*

**15.30-16.00 Lecture**

*Chair: Sebastiano Andò (Cosenza)*

Journey to success: career pathways for biomedical scientists in pathology and laboratory medicine

*Mark E. Sobel (Bethesda)*

**16.00-18.00 Session I: Cancer drug resistance**

*Chair: Daniela Quaglino (Modena)*

Gamma-glutamyltransferase activity of melanoma cells: tumor progression and cancer drug resistance

*Alessandro Corti (Pisa)*

Overcoming resistance to EGFR tyrosine kinase inhibitors by novel therapeutic strategies in non small lung cancer cell lines

*Andrea Cavazzoni (Parma)*

Novel therapeutic approaches in chronic myeloid leukemia: role of survivin and irf-5

*Elena Tirrò (Catania)*

Estrogen receptor signaling in breast cancer: endocrine resistance and new therapeutic opportunities

*Ines Barone (Cosenza)*

**18.00-18.15 COFFEE BREAK**

**18.15- 18.45 Lecture**

*Chair: Giancarlo Vecchio (Naples)*

Cancer stem cells and cell cycle

*Richard G. Pestell (Philadelphia)*

**18.45-20.15 Session II: Oxidative Stress**

*Chair: Angelo Messina (Catania)*

Reactive oxygen species, hypoxia and hepatic myofibroblasts in chronic liver diseases  
*Erica Novo (Turin)*

Cholesterol oxidation and inflammatory molecules in atherosclerosis  
*Paola Gamba (Turin)*

Oxidative stress and PKC signaling in brain tumors  
*Barbara Marengo (Genoa)*

## **3th December, 2009**

### **9.00-11.00 Session III: Multifaceted Action of Chemokines**

*Chair: Anna Gasperi-Campani (Bologna)*

Chemokine degradation by local expression of the chemokine decoy receptor D6 inhibits tumor growth

*Benedetta Savino (Milan)*

A key pathway in immune responses: the modulatory role of leptin

*Carlo Procaccini (Naples)*

Protection against inflammation- and autoantibody-caused fetal loss by the chemokine decoy receptor D6

*Elena Monica Borroni (Milan)*

Modifications of the immune system in elderly people and age-related diseases:

Alzheimer's disease, a typical disease of "unsuccessfully aged" people

*Silvio Buffa (Palermo)*

### **11.00-11.15 COFFEE BREAK**

### **11.15-12.45 Session IV: Novel therapeutic targets and strategies in human disease**

*Chair: Giuseppe Poli (Turin)*

Non-transcriptional control of DNA replication by c-myc: novel mechanism for its oncogenic function

*Luca Ruggiero (Milan)*

Analysis of genome-wide gene expression data from hormone-responsive human breast cancer cells

*Maria Ravo (Naples)*

Natural lipids and antimycobacterial activity

*Emanuela Greco (Rome)*

### **12.45-14.00 LUNCH**

**14.00-16.30 Session IV: Novel therapeutic targets and strategies in human disease**

*Chair: Nicola Schiavone (Florence)*

Antiangiogenic activities of chemopreventive drugs  
*Rosaria Cammarota (Varese)*

Urokinase-plasminogen activator receptor signaling in tumor growth and metastasis  
*Francesca Margheri (Florence)*

Novel pathways and pharmacological tools in cellular autophagy: implications for mitochondrial quality control  
*Michelangelo Campanella (London)*

Is leptin signaling a new therapeutic target for breast cancer?  
*Viviana Bartella (Cosenza)*

Gene therapy of cystic fibrosis  
*Stefano Castellani (Foggia)*

**16.30 Concluding remarks**

*Sebastiano Andò (Cosenza)*

# **ABSTRACTS**

## ABSTRACTS LIST

### Lecture

**Journey to success: career pathways for biomedical scientists in pathology and laboratory medicine**

**Mark E. Sobel, MD, PhD**

*Executive Officer, American Society for Investigative Pathology.*

### Session I: Cancer drug resistance

**Gamma-glutamyltransferase activity of melanoma cells: tumor progression and cancer drug resistance**

**Corti A, Giommarelli C<sup>1</sup>, Dilda P<sup>2</sup>, Paolicchi A, Zunino F<sup>1</sup>, Hogg PJ<sup>1</sup> and Pompella A**  
*Dept. of Experimental Pathology, University of Pisa (Italy), (1) Preclinical Chemotherapy and Pharmacology Unit, Fondazione IRCCS Istituto Nazionale Tumori, Milano (Italy), (2) UNSW Cancer Research Centre, University of New South Wales, Sydney (Australia).*

**Overcoming resistance to EGFR tyrosine kinase inhibitors by novel therapeutic strategies in non small lung cancer cell lines**

**Cavazzoni A.<sup>1</sup>, Alfieri R.R.<sup>1</sup>, Galetti M.<sup>1</sup>, La Monica S.<sup>1</sup>, Bonelli M.<sup>1</sup>, Fumarola C.<sup>1</sup>, Lodola A.<sup>2</sup>, Carmi C.<sup>2</sup>, Goldoni M.<sup>3</sup>, Tagliaferri S.<sup>3</sup>, Mutti A.<sup>3</sup>, Generali D.<sup>4</sup>, Tiseo M.<sup>5</sup>, Mor M.<sup>2</sup>, Ardizzone A.<sup>5</sup>, Petronini P.G.<sup>1</sup>**

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**Novel therapeutic approaches in chronic myeloid leukemia: role of survivin and irf-5**

**Elena Tirò, Michele Massimino, Stefania Stella, Pietro Buffa, Livia Manzella, Paolo Vigneri and Angelo Messina**

*Department of Biomedical Sciences, University of Catania, Catania, Italy.*

**Estrogen receptor signaling in breast cancer: endocrine resistance and new therapeutic opportunities**

**Ines Barone, PhD<sup>1,2</sup>, Stefania Catalano, MD<sup>2,3</sup>, Cinzia Giordano, PhD<sup>2,3</sup>, Suzanne A. W. Fuqua, PhD<sup>1</sup>, and Sebastiano Andò, MD<sup>2,4</sup>.**

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## **Session II: Oxidative Stress**

**Reactive oxygen species, hypoxia and hepatic myofibroblasts in chronic liver diseases**

**Erica Novo** and Maurizio Parola

*Dipartimento di Medicina e Oncologia Sperimentale – Università di Torino.*

**Cholesterol oxidation and inflammation in atherosclerosis**

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**Oxidative stress and PKC signaling in neuroblastoma cells**

**Barbara Marengo**, Chiara De Ciucis, Roberta Ricciarelli, Mariapaola Nitti, Umberto M. Marinari, Maria A. Pronzato, Cinzia Domenicotti

*Department of Experimental Medicine, Section of General Pathology, University of Genoa, Italy.*

## **Session III: Multifaceted Action of Chemokines**

**Chemokine degradation by local expression of the chemokine decoy receptor D6 inhibits tumor growth**

**Benedetta Savino**<sup>1,2</sup>, Elena Monica Borroni<sup>1,2</sup>, Achille Anselmo<sup>2</sup>, Alberto Mantovani<sup>1,2</sup>, Massimo Locati<sup>1,2</sup>, Raffaella Bonecchi<sup>1,2</sup>

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**A key pathway in immune responses: the modulatory role of leptin**

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**Modifications of the immune system in elderly people and age-related diseases:  
Alzheimer's disease, a typical disease of "unsuccessfully aged" people**

**Silvio Buffa<sup>1</sup>, Matteo Bulati<sup>1</sup>, Mariavaleria Pellicanò<sup>1</sup>, Anna Di Prima<sup>2</sup> Sonya Vasto<sup>1</sup>,  
Giuseppina Candore<sup>1</sup>, Giuseppina Colonna-Romano<sup>1</sup>, Calogero Caruso<sup>1</sup>**

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**Session IV: Novel therapeutic targets and strategies in human disease**

**Non-transcriptional control of DNA replication by c-myc: novel mechanism for its  
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**David Dominguez-Sola<sup>1\*</sup>, Carol Y. Ying<sup>1\*</sup>, Carla Grandori<sup>2#</sup>, Luca Ruggiero<sup>1Φ</sup>,  
Brenden Chen<sup>1</sup>, Muyang Li<sup>1</sup>, Denise A. Galloway<sup>2</sup>, Wei Gu<sup>1</sup>, Jean Gautier<sup>1\*</sup> &  
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**Genome-wide analysis of estrogen signaling in hormone-responsive human breast  
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**Maria Ravo<sup>1</sup>, Oli M.V. Grober<sup>1</sup>, Lorenzo Ferraro<sup>1,2</sup>, Ornella Paris<sup>1,2</sup>, Roberta Tarallo<sup>1</sup>,  
Margherita Mutarelli<sup>1,3</sup>, Luigi Cicatiello<sup>1</sup> and Alessandro Weisz<sup>1,2,4</sup>**

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**Emanuela Greco<sup>a</sup>, Marilina B. Santucci<sup>a</sup>, Gianluca Quintiliani<sup>a</sup>, Massimiliano Papi<sup>b</sup>,  
Marco De Spirito<sup>b</sup>, Vittorio Coalizzi<sup>a</sup>, Maurizio Fraziano<sup>a</sup>**

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**Antiangiogenic activities of chemopreventive drugs**

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**Urokinase-plasminogen activator receptor signalling in tumor growth and  
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**Francesca Margheri, Simona Serrati, Anastasia Chillà, Marco Pucci, Gabriella Fibbi  
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*Department of Experimental Pathology and Oncology, University of Florence.*

**Novel pathways and pharmacological tools in cellular autophagy: implications for mitochondrial quality control**

Annalisa Gastaldello<sup>1</sup>, Priya Gami<sup>1</sup>, Holly Callaghan<sup>1</sup>, Teresa Lyndon-Kennedy<sup>1</sup> and Michelangelo Campanella<sup>1,2</sup>

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**Is leptin signaling a new therapeutic target for breast cancer?**

Viviana Bartella, Stefania Catalano, Loredana Mauro, Maria Luisa Panno, Eva Surmacz and Sebastiano Andò

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**Gene therapy of cystic fibrosis**

Stefano Castellani, Sante Di Gioia, Massimo Conese

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*Lecture*

## **Journey to success: career pathways for biomedical scientists in pathology and laboratory medicine**

**Mark E. Sobel, MD, PhD**

*Executive Officer, American Society for Investigative Pathology*

Pathology is at the interface of basic science, translational research, and clinical care. Modern health care needs physician scientists in pathology and laboratory medicine. A biomedical scientist in pathology and/or laboratory medicine is a person who is scientifically trained in the life sciences to investigate the pathogenesis of human disease. Scientific training entails acquiring a doctoral (PhD or ScD) degree in one of the basic sciences, or acquiring a medical (MD) degree or equivalent (such as a degree in osteopathic medicine or veterinary medicine). There are many possible pathways to satisfy the intellectual curiosity of a biomedical scientist engaged in the general discipline of pathology. Traditional paths include an academic position as a principal investigator of a research laboratory or an academic position as a clinical care professional. Alternate pathways include career opportunities in the pharmaceutical industry, biotechnology, or clinical diagnostics, or non-traditional careers in scientific writing, teaching, public policy, or patent law.

*Session I: Cancer drug resistance*

## **Gamma-glutamyltransferase activity of melanoma cells: tumor progression and cancer drug resistance**

**Corti A, Giommarelli C<sup>1</sup>, Dilda P<sup>2</sup>, Paolicchi A, Zunino F<sup>1</sup>, Hogg PJ<sup>1</sup> and Pompella A**

*Dept. of Experimental Pathology, University of Pisa (Italy), (1) Preclinical Chemotherapy and Pharmacology Unit, Fondazione IRCCS Istituto Nazionale Tumori, Milano (Italy), (2) UNSW Cancer Research Centre, University of New South Wales, Sydney (Australia).*

Gamma-glutamyltransferase (GGT) is a key enzyme involved in GSH metabolism, whose expression is often significantly increased in human malignancies. In the past years several studies focused on the possible role of GGT in tumor progression, invasion and drug resistance, and the involvement of a pro-oxidant activity of GGT – besides its early recognized contributions to cellular antioxidant defences – has been repeatedly documented. It was shown that GGT-derived pro-oxidants can modulate important redox-sensitive processes such those mediated by NF- $\kappa$ B and AP-1, with possible effects on proliferative/apoptotic balance and with important implications in tumour progression and drug resistance (1).

In agreement with these data, our studies documented the effects of GGT-derived pro-oxidants on DNA damage in melanoma cells, both in normal culture conditions and in the presence of added GGT substrates, thus suggesting a role of GGT expression *per se* in tumor progression (2). In this perspective, we hypothesized that the low but persistent oxidative stress produced by GGT activity could also produce an antioxidant adaptation of cancer cells against oxidative stress, which itself is a factor of resistance against the effects of prooxidant drugs. Indeed GGT expressing melanoma cells were shown to be more resistant against arsenic trioxide, possibly thanks to the higher levels of catalase expression (3, 4). In addition, GGT-derived thiol metabolites were also shown to play a role in extracellular detoxification/resistance of melanoma cells against cisplatin (CDDP), leading to formation of adducts which are far less cell-permeable (5).

Due to the high specificity of GGT for gamma-glutamyl compounds, the possible exploitation of GGT as a target for cancer therapies through the use of gamma-glutamyl pro-drugs was also investigated. In particular, 4-[N-(S-glutathionyl-acetyl)amino] phenylarsinous acid (GSAO) – a glutathione-S conjugate of aminophenylarsonous acid, was found to display an antiproliferative activity strongly correlated with the level of cell surface GGT (6).

The envisaged roles of GGT activity in tumor progression/resistance phenotype of cancer cells suggests the potential advantages of GGT-targeted therapies – e.g. GGT inhibitors, gamma-glutamyl pro-drugs – or the association of more agents in therapy in order to overcome GGT-mediated resistance. Future studies will substantiate the applicability and usefulness of such approaches in therapy.

## **References**

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*Session I: Cancer drug resistance*

## **Overcoming resistance to EGFR tyrosine kinase inhibitors by novel therapeutic strategies in non small lung cancer cell lines**

**Cavazzoni A.<sup>1</sup>, Alfieri R.R.<sup>1</sup>, Galetti M.<sup>1</sup>, La Monica S.<sup>1</sup>, Bonelli M.<sup>1</sup>, Fumarola C.<sup>1</sup>, Lodola A.<sup>2</sup>, Carmi C.<sup>2</sup>, Goldoni M.<sup>3</sup>, Tagliaferri S.<sup>3</sup>, Mutti A.<sup>3</sup>, Generali D.<sup>4</sup>, Tiseo M.<sup>5</sup>, Mor M.<sup>2</sup>, Ardizzone A.<sup>5</sup>, Petronini P.G.<sup>1</sup>**

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Epidermal Growth Factor Receptor (EGFR) is an established new target for the treatment of epithelial tumors, including non-small cell lung cancer (NSCLC). Small molecules inhibitors, such as erlotinib and gefitinib, have been proven to be a useful addition to standard therapy in advanced NSCLC. However, tumor cells often acquire resistance to these EGFR inhibitors. The mechanisms that mediate resistance to gefitinib treatment include secondary mutations in EGFR i.e. the point mutation T790M, the amplification of MET, constitutive or alternative activation of downstream pathways and efflux of the drug from the cells.

In this study we have investigated in a panel of NSCLC cell lines the mechanisms underlying primary or acquired resistance to gefitinib and new therapeutic approaches to circumvent gefitinib resistance.

Clear evidence exists for the involvement of constitutive activation of the PI3K/AKT signaling pathway in lung carcinogenesis and in resistance to tyrosine-kinase inhibitors. We assessed the effects of combining the mTOR inhibitor everolimus (RAD001) with gefitinib on a panel of NSCLC cell lines characterized by gefitinib-resistance and able to maintain pS6K phosphorylation after gefitinib treatment. Everolimus plus gefitinib induced a significant decrease in the activation of MAPK and mTOR signaling pathways downstream of EGFR and resulted in a growth-inhibitory effect rather than in an enhancement of cell death. A synergistic effect was observed in those cell lines characterized by high proliferative index and low doubling time.

The design and synthesis of new tyrosine kinase inhibitors is another rational approach to treat acquired resistance to gefitinib. In the last few years we focused on the design and synthesis of a new generation of EGFR reversible and irreversible inhibitors with an improved in vitro potency and selectivity. We identify a novel EGFR tyrosine kinase inhibitor, the 5-benzylidene-hydantoin UPR1024, able of both blocking EGFR tyrosine kinase activity and inducing genomic DNA damage. Moreover, we are now extending the chemical diversity of new irreversible EGFR inhibitors.

One of the proposed mechanism of resistance to gefitinib and other tyrosine kinase inhibitors is related to low level of intracellular drug concentration. In addition to the transporters involved in drug efflux, a number of transporters are responsible for drug uptake.

In the last part of our research we have characterized the gefitinib transport system and we have evaluated whether the intracellular concentration of gefitinib may affect the EGFR autophosphorylation.

*Session I: Cancer drug resistance*

## **Novel therapeutic approaches in chronic myeloid leukemia: role of survivin and irf-5**

**Elena Tirò, Michele Massimino, Stefania Stella, Pietro Buffa, Livia Manzella, Paolo Vigneri and Angelo Messina**

*Department of Biomedical Sciences, University of Catania, Catania, Italy*

Chronic Myelogenous Leukemia (CML) is a clonal disorder of myeloid progenitor cells caused by a translocation between chromosomes 9 and 22 that generates the abnormal Philadelphia chromosome. The ensuing chimaeric oncogene *BCR-ABL* encodes for a cytoplasmic protein with constitutive tyrosine kinase activity.

Survivin is a member of the inhibitor of apoptosis proteins that is highly expressed in most forms of human cancer and reduces the sensitivity of neoplastic cells to multiple death stimuli.

Interferon Regulatory Factor 5 (IRF-5), is a transcription factor, which modulates the immune response against viral infections and cancer and displays tumor suppressor-like properties.

Aim of this research was to study the possible relationships of the BCR-ABL oncoprotein with Survivin and IRF-5.

Using murine and human CML cells, we found that BCR-ABL up-regulated Survivin expression through the JAK2/STAT3 pathway. We also observed that Survivin silencing significantly increased cell death in CML cells exposed to hydroxyurea (HU) but failed to restore Imatinib Mesylate (IM) sensitivity in IM-resistant cells. This data indicated that Survivin lowered the sensitivity of CML cells to different apoptotic stimuli but was not directly involved in the development of IM resistance. However, down-regulation of Survivin strongly increased HU-mediated killing of cells unresponsive to IM because of point mutations in the BCR-ABL kinase domain. Finally, incubation of IM-resistant cells with cell-permeable shepherdin, an inhibitor of heat shock protein 90 that reduces Survivin expression, also enhanced HU-induced death. These findings suggest that strategies aimed at reducing Survivin expression sensitize IM-resistant CML cells to the cytotoxic effect of HU.

As for the relationship between BCR-ABL and IRF-5, we found that IRF-5 was expressed in CML cells and co-immunoprecipitated with BCR-ABL. In CML cells, IRF-5 was mostly confined to the cytoplasm. However, treatment with Interferon (IFN) or IM relocalized IRF-5 to the nucleus. The latter finding suggested that BCR-ABL-dependent phosphorylation influenced IRF-5 subcellular localization. Mutagenesis to phenylalanine of tyrosine 104 in the IRF-5 sequence produced an IRF-5 variant that displayed reduced levels of tyrosine phosphorylation. In addition, the IRF-5Y104F mutant mainly localized to the cell nucleus, confirming that tyrosine-phosphorylated IRF-5 was preferentially cytoplasmic. Finally, over-expression of IRF-5 Y104F led to a significant reduction of foci formation in CML cells. These results imply that disrupting the interaction between IRF-5 and BCR-ABL by IFN or IM treatment may contribute to the clinical efficacy of these drugs on CML cells.

*Session I: Cancer drug resistance*

## **Estrogen receptor signaling in breast cancer: endocrine resistance and new therapeutic opportunities**

**Ines Barone, PhD<sup>1,2</sup>, Stefania Catalano, MD<sup>2,3</sup>, Cinzia Giordano, PhD<sup>2,3</sup>, Suzanne A. W. Fuqua, PhD<sup>1</sup>, and Sebastiano Andò, MD<sup>2,4</sup>.**

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The standard treatment for ER $\alpha$ -positive breast cancer includes two strategies to reduce the effects of estrogens on tumor growth, either by blocking estrogen binding, using competitive inhibitors such as tamoxifen, or inhibiting endogenous estrogen production using aromatase inhibitors (AIs), such as letrozole, anastrozole, or exemestane. However, many ER $\alpha$ -positive breast tumors initially fail to respond (*de novo* resistance), or resistance develops during treatment leading to disease progression (acquired resistance).

There are relatively few mutations which have been reported in the ER $\alpha$  gene, surprising since many therapeutic resistance mechanisms involve mutation of the target. We have previously identified a lysine to arginine transition at residue 303 (K303R) in ER $\alpha$  in premalignant breast lesions and invasive breast cancers, which confers estrogen hypersensitivity. We hypothesized that such a mutant could provide a continuous mitogenic stimulus to the breast even during phases of low circulating hormone, such as menopause, thus affording a proliferative advantage especially during treatment with AIs. As preclinical models to test this possibility, we generated K303R-overexpressing MCF-7 cells stably transfected with an aromatase expression vector, and found that the mutant-expressing cells were resistant to the inhibitory effect of Anastrozole on growth. We demonstrated that AI resistance arises through an enhanced cross-talk of the IGF-1R/IRS-1/Akt pathway with ER $\alpha$ , and the serine (S) residue 305 adjacent to the K303R mutation plays a key role in mediating this cross-talk. The role of S305 phosphorylation in AI<sup>R</sup> represents a novel mechanism and we speculate that blockade of this phosphorylation site may be a new therapeutic strategy to block ER $\alpha$  hyperactivation and overcome resistance in mutation-positive patients that are resistant to aromatase inhibitor therapy.

The possibility to combine endocrine therapy with novel signal transduction inhibitors (STI) is also suggested by our recent findings showing a new molecular mechanism by which 17- $\beta$ estradiol (E<sub>2</sub>), through an enhanced cross talk between GF-Rs, c-Src, and ER signaling, can phosphorylate and thus rapidly activate aromatase. This results in a positive nongenomic autocrine loop between E<sub>2</sub> and aromatase in breast cancer cells. *In vivo* labeling experiments and site-directed mutagenesis studies demonstrated that phosphorylation of the 361-tyrosine residue is crucial in the up-regulation of aromatase activity under E<sub>2</sub> exposure.

Our progress in understanding ER biology and aromatase function has revealed complex signaling interaction between these proteins and other signal transduction pathways, suggesting novel molecular biomarkers and new therapeutic options to treat breast cancer.

## **Reactive oxygen species, hypoxia and hepatic myofibroblasts in chronic liver diseases**

**Erica Novo** and Maurizio Parola

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Fibrotic progression of chronic liver diseases (CLDs) of different aetiology to the common advanced-stage of cirrhosis can be envisaged as a dynamic and highly integrated cellular response to chronic liver injury. Liver fibrosis is accompanied by perpetuation of liver injury, chronic hepatitis and persisting activation of tissue repair mechanisms, leading eventually to excess deposition of ECM components. Liver fibrogenesis (i.e., the process) is sustained by populations of highly proliferative, pro-fibrogenic and contractile myofibroblast-like cells (MFs) that, according to current literature, can originate mainly from hepatic stellate cells (HSC) or, to a less extent, from portal fibroblasts, bone marrow – derived cells or by a process of epithelial to mesenchymal transition involving hepatocytes or cholangiocytes. In the scenario of chronic liver diseases, irrespective of aetiology, oxidative stress, hypoxia and angiogenesis are emerging as major pathogenic event mechanistically involved in fibrogenesis progression.

Oxidative stress, through the generation of reactive oxygen species (ROS) as well as other redox-related reactive intermediates, has been shown to modulate phenotypic responses of human activated- and MF-like HSC (HSC/MFs), including proliferation, increased synthesis of collagen type I and of components related to extracellular matrix (ECM) remodeling as well as chemotaxis. The analysis of redox – mediated responses also revealed that human HSC/MFs can survive to severe oxidative stress as well as to the exposure to other pro-apoptotic stimuli. This survival attitude of human HSC/MFs mechanistically involves up-regulation of bcl-2 expression, a key feature also observed “*in vivo*” in liver specimens from cirrhotic HCV patients that can favour fibrogenesis and is lost with HSC/MFs senescence.

Recently, data from either clinical or experimental conditions of CLDs have suggested that hepatic angiogenesis, a hypoxia – dependent process leading to the formation of new vessels from pre-existing ones, may play a pathogenic role by favour fibrogenesis progression towards cirrhosis. A series of related “*in vivo*” and “*in vitro*” studies revealed that hypoxia is crucial in up-regulating expression of pro-angiogenic cytokines also in HSC/MFs suggesting two concepts: a) VEGF and Ang-1 may contribute to fibrogenesis by acting as hypoxia-inducible, autocrine, and paracrine factors able to recruit myofibroblast-like cells; b) HSC/MFs, in addition to their established pro-fibrogenic role, may also contribute to neo-angiogenesis during chronic hepatic wound healing.

Finally, by analyzing the molecular mechanisms able to sustain migration of human HSC/MFs, we found that chemotaxis stimulated by polypeptides like PDGF-BB, VEGF and MCP-1 or by hypoxic conditions critically requires a NADPH-oxidase- or mitochondrial-dependent, respectively, increased generation of intracellular ROS and the consequent activation of JNK1/2 isoforms in addition to activation of Ras/ERK signalling. Moreover, a polypeptide-independent rise in intracellular ROS is sufficient

to induce migration/chemotaxis of HSC/MFs, suggesting that in the CLD scenario, ROS released by damaged hepatocytes or activated inflammatory cells may trigger the same response. These data outline a putative therapeutic target for antioxidants or small molecules like tyrosine kinase inhibitors.

The search for molecular mechanisms and signalling pathways involved in the pro-fibrogenic responses of HSC/MFs is currently emerging as the necessary base of knowledge to properly design and evaluate new and selective therapeutic strategies able to significantly affect the progression of fibrogenesis towards the end-point of cirrhosis.

## Cholesterol oxidation and inflammation in atherosclerosis

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Cholesterol oxidation products (oxysterols) represent a family of 27-carbon cholesterol oxidation derivatives that may be absorbed either by the diet or originated endogenously. They are considered as potentially involved in the progression of atherosclerotic lesions because they are able to modulate several biochemical effects promoting chronic inflammation, fibrosis and programmed cell death. The importance of these compounds is sustained by the observed correlation between the quantity of total oxysterols and the respective total cholesterol amount recovered in human atherosclerotic plaques.

The aim of our research was to evaluate the potential role of oxysterols in the development of the atherosclerotic lesion, studying various key steps of this complex process, including fibrogenesis, monocyte recruitment into the subintimal space and their differentiation into macrophages with consequent foam cells formation. U937 human promonocytic cells were used as monocyte model system and treated with a biologically representative oxysterol mixture, like that detectable in hypercholesterolemic individuals, which was shown to be able to sustain a chronic inflammatory process by up-regulating the expression of various pro-inflammatory genes. In particular we focused our attention on the transforming growth factor  $\beta$ 1 (TGF $\beta$ 1), a very efficient fibrogenic cytochicine, on the monocyte chemotactic protein-1 (MCP-1) and on CD36 scavenger receptor.

Of note, oxysterols have been shown to significantly contribute to the vascular remodelling occurring in atherosclerosis, as they lead to a marked over-expression of TGF $\beta$ 1 but also MCP-1 and CD36. On the contrary, unoxidized cholesterol did not exert any significant modulation of the investigated genes, pointing to oxidation as a crucial reaction for the sterol to become proatherogenic. Interestingly, the lack of direct toxicity against vascular cells by the oxysterol mixture may significantly facilitate their ability to over-express genes related to inflammation.

The investigation of the molecular signalling modulated by oxysterols allowed to demonstrate, by means of both metabolic inhibitors and small interfering RNA technology, the involvement of G proteins, Src, phospholipase C, PKC $\delta$  and ERK phosphorylation pathways, as well as that of redox-sensitive transcription factors like NF- $\kappa$ B and PPAR $\gamma$ .

## **Oxidative stress and PKC signaling in neuroblastoma cells**

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Reactive oxygen species (ROS) are considered as DNA damaging agents that increase the mutation rate and promote oncogenic transformation. However, in contrast with their pathological role, intracellular ROS can act as second messenger molecules in signalling cascades, controlling diverse cellular events such as cell differentiation, proliferation and death.

In fact, free radicals derived from cell exposure to radiations and chemotherapeutic agents, modulate the activity of several signalling proteins, such as MAP kinases and protein kinase C (PKC) isoforms. Activation of PKCs depends on the oxidation of cysteine residues, present both in regulatory and catalytic domains. In particular, PKC  $\delta$ , a major isoform ubiquitously expressed, is known for its apoptotic properties and is involved in the cellular response to oxidative stress. Our previous studies demonstrated that neuroblastoma (NB) cell death, triggered by L-buthionine-S,R-sulfoximine (BSO), a glutathione (GSH)-depleting agent, is mediated by ROS over-production, PKC  $\alpha$  inactivation and PKC  $\delta$  translocation to mitochondria.

These findings suggested further studies on different NB cell lines. MYCN amplified cells (SK-N-BE-2-C and LAN5) were found to be more sensitive to BSO than non-amplified cells (SHSY5Y, ACN and GIMEN). BSO-induced oxidative stress, accompanied by an increase in superoxide dismutase 1 (SOD-1) activity, caused DNA damage and apoptosis in MYCN amplified cells.

More recently, the three non-amplified NB cells were transiently transfected with PKC  $\delta$  and grown in the presence of BSO. Over-expression of the  $\delta$  isoenzyme increased ROS production, DNA oxidation and the apoptotic rate, these cellular responses being over-stimulated after BSO administration. A key role of NADPH oxidase was demonstrated by the suppression of PKC-induced cell responses in NB cells pre-treated with diphenylene-iodonium (DPI), a NADPH oxidase inhibitor. Moreover, the essential role of the  $\delta$  isoform in the commitment to apoptosis was confirmed by the enhanced sensitivity of PKC over-expressing cells to etoposide (a drug commonly used in NB therapy).

Collectively, our data provides evidence that PKC  $\delta$ , in combination with chemotherapeutic agents, could act as a chemo-sensitiser for reducing drug dosage and relative periods of treatment, in addition to preventing the development of chemo-resistance.

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*Session III: Multifaceted Action of Chemokines*

**Chemokine degradation by local expression of the chemokine decoy receptor D6 inhibits tumor growth.**

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Tumor initiation and progression are multifactorial processes, regulated by intrinsic genetic changes and microenvironmental factors. Among these factors members of the chemokine superfamily play a key role. Chemokines and their receptors have been demonstrated to be expressed in most primary tumors and in metastatic sites, by tumoral cells and by non-neoplastic “host” cells that cooperate and control tumor biology. Chemokines may act directly on tumoral cells controlling their growth and local invasion and directing their homing to specific metastatic sites.

Chemokine biological activities are mediated by specific cell surface seven transmembrane receptors coupled with heterotrimeric G proteins that after ligand engagement activate intracellular signaling cascades that exit in cell migration. Chemokine receptors with structural features that are inconsistent with a signaling function have been described. These atypical chemokines receptors are considered “silent” because after chemokine engagement they do not directly mediate migration or other conventional signaling, but regulate immune responses by acting as decoy and scavenger. In addition to be involved in the resolution phase of inflammatory response, several data clearly indicate that these atypical chemokine receptors have an important role in tumor biology regulating the intra-tumoral amount of chemokines and the tumor infiltrate.

The chemokine decoy receptor D6 binds most of the inflammatory CC chemokines and it is extremely efficient in targeting chemokines to degradation. In vivo models with D6-/ mice have highlighted its important role in the resolution of inflammation and in the protection from inflammation-driven carcinogenesis. D6 is expressed at high levels by lymphatic vessels endothelium and its expression was reported also by some vascular tumors. Here we report that D6 is also expressed by HHV8-infected spindle cells of Kaposi’s Sarcoma (KS) lesions. To address D6 role in KS biology, KS-Imm human Kaposi’s sarcoma cells overexpressing D6-EGFP chimeric protein were studied *in vitro* and *in vivo*. *In vitro*, D6 expression did not influence cell proliferation while when KS-Imm/D6 cells were subcutaneously injected in the flank of CD-1 nude mice showed significantly reduced growth compared to mock transfected cells. FACS analysis showed that these tumors had limited leukocyte infiltration, including granulocytes and macrophages. These data suggest that D6, through its scavenging activity, regulate the bioavailability of inflammatory chemokines and therefore controls leukocyte accumulation at tumor site that support tumor growth and angiogenesis.

### *Session III: Multifaceted Action of Chemokines*

## **A key pathway in immune responses: the modulatory role of leptin**

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### **Background:**

Leptin is a 16 kDa non-glycosylated hormone with structural similarities to the cytokines of the type I cytokine family that includes the growth hormone, prolactin, erythropoietin, interleukin (IL)-3, IL-11, IL-12, oncostatin M, and granulocyte-colony stimulating factor.

Since leptin is mainly produced by adipocytes in proportion to fat size stores, it was originally thought that the activity of leptin was to regulate the balance between food intake and energy expenditure, signalling to the brain the changes in stored energy. However, both leptin-deficient (*ob/ob*) and leptin receptor-deficient (*db/db*) mice are not only severely obese but also display marked abnormalities in the neuroendocrine function, angiogenesis, reproduction, insulin secretion, wound healing, hematopoiesis and lymphopoiesis, suggesting that leptin may exerts pleiotropic effects on different tissues. More in detail, in our laboratory we demonstrated that leptin promotes the proliferation and IL-2 secretion by naïve T cells, while on memory T cells, leptin promotes the switch towards T helper (Th)1-cell immune responses by increasing interferon-γ (IFN-γ) and TNF-α secretion, IgG2a production from B cells, and by promoting delayed-type hypersensitivity (DTH) responses. The immunomodulatory effects of leptin have also been linked to enhanced susceptibility to different autoimmune disease, such as experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. Indeed we demonstrated that leptin is involved in the induction and progression of EAE, since *ob/ob* mice are resistant to EAE induction, with increased IL-4 and a lack of IFN-γ production after myelin-specific stimulation of T cells. Interestingly, leptin replacement renders these mice susceptible to the disease, while *in vivo* abrogation of leptin with anti-leptin Abs, either before or after onset of EAE, improved clinical score, slowed disease progression, reduced disease relapses, inhibited PLP(139-151)-specific T cell proliferation, and switched cytokine secretion toward a Th2/regulatory profile. Moreover in human studies it has been also shown that leptin production was significantly increased in both serum and CSF(cerebrospinal fluid) of RRMS (relapsing-remitting multiple sclerosis) patients and positively correlated with IFN-gamma secretion in the CSF.

### **Current research:**

Since we found in RRMS patients an inverse correlation between leptin secretion and the frequency of T reg cells, a well known cellular subset able to dampen autoimmune response, we recently focused our attention on the role of leptin in the modulation of this cellular population, that is anergic to TCR-mediated stimulation. We show that

freshly isolated T reg cell produced leptin, that is responsible for an autocrine inhibitory loop that constrains their expansion, and expressed high amounts of leptin receptor (ObR). *In vitro* neutralization with leptin monoclonal antibody (mAb), during anti-CD3 and anti-CD28 stimulation, resulted in T reg cells proliferation, as indicated by downmodulation of the cyclin-dependent kinase inhibitor p27 ( $p27^{kip1}$ ), and the phosphorylation of the extracellular-related kinases 1 (ERK1) and ERK2 and this phenomenon was interleukin-2 (IL-2) dependent. Moreover mouse Treg cells expanded *in vivo* more robustly and earlier when adoptively transferred into leptin-neutralized WT mice or into leptin-deficient *ob/ob* mice. So, taken together, these results suggest that leptin can act as a negative signal for the proliferation of T reg cells.

**Possible future therapeutic approach:**

Given the importance of T reg cells in the mechanisms of immune regulation and their protective role in several autoimmune conditions, our results, indicating that leptin neutralization can “unlock” the hyporesponsiveness of T reg cells, might so describe a novel strategy to expand human peripheral T reg cells and therefore envision new possibilities of anti-leptin-based approaches for the immunotherapy of conditions characterized by low numbers of these cells, such as autoimmune disorders.

*Session III: Multifaceted Action of Chemokines*

**Protection against inflammation- and autoantibody-caused fetal loss by the chemokine decoy receptor D6.**

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Fetal loss in animals and humans is frequently associated with inflammatory conditions. During inflammation, leukocyte recruitment is a tightly regulated multistep process involving adhesion molecules and locally produced soluble mediators. Among these, chemokines are essential for leukocyte migration and activation. This large family of cytokines has been classified into four subfamilies (CXC, CC, CX3C, and C) according to the relative position of cysteine residues, and in homeostatic (i.e., produced constitutively) and inflammatory (i.e., produced in response to inflammatory or immunological stimuli) categories according to their production. Biological activities are mediated by a subfamily of chemoattractant receptors belonging to the large family of seven transmembrane domain G protein-coupled receptors. Chemokines also interact with a distinct group of “silent” receptors with structural similarity with conventional receptors but lacking signaling function, which includes the Duffy antigen receptor for chemokines (DARC), CCX-CKR, and D6. D6 binds most inflammatory, but not homeostatic, CC chemokines, internalizes constitutively, and targets the ligand for degradation. Differently from other chemokine receptors, D6 expression has been reported mainly in nonhematopoietic cells and includes endothelial cells lining afferent lymphatic in certain anatomical sites, such as skin, gut, and lung. Evidence from gene-targeted animals indicates that D6 plays a nonredundant role in the control of the local inflammatory response by acting as a decoy and scavenger receptor for inflammatory chemokines in the skin. D6 expression has also been detected in placenta, but its cellular location and function in this organ have not been previously investigated. D6 is expressed in placenta on invading extravillous trophoblasts and on the apical side of syncytiotrophoblast cells, at the very interface between maternal blood and fetus. Exposure of D6<sup>-/-</sup> pregnant mice to LPS or antiphospholipid autoantibodies results in higher levels of inflammatory CC chemokines and increased leukocyte infiltrate in placenta, causing an increased rate of fetal loss, which is prevented by blocking inflammatory chemokines. Thus, the promiscuous decoy receptor for inflammatory CC chemokines D6 plays a nonredundant role in the protection against fetal loss caused by systemic inflammation and antiphospholipid antibodies.

### *Session III: Multifaceted Action of Chemokines*

## **Modifications of the immune system in elderly people and age-related diseases: Alzheimer's disease, a typical disease of “unsuccessfully aged” people.**

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Ageing is a complex multifactorial process due to the accumulation of changes that induce modifications in performances of most physiological systems and increases susceptibility to diseases and death. Ageing progression causes a declining ability to respond to environmental stimuli, increasing homeostatic imbalance and increases risk of disease. Many events occur during this process, such as the modification of the immune system, known as Immunosenescence, that involves both innate and instructive immunity, the establishment of a chronic low-grade inflammatory state and an increase in production of pro-inflammatory cytokines and chemokines. Moreover, many scientific studies that have established the role of immunogenetics in successful (centenarians) and unsuccessful ageing, support the idea that longevity is based on genetic background.

We have focused our attention on Alzheimer's disease, a typical disease of “unsuccessfully aged” people. The role of immune-inflammatory processes may be particularly relevant in this age-related disease, indeed microglia, astrocyte and neurones are responsible for the local inflammatory reaction. Activated cells strongly produce inflammatory mediators as pro-inflammatory cytokines (IL-1  $\beta$ , IL-6, TNF- $\alpha$ ) and chemokines (IL-8, MIP-1  $\alpha$ , MCP-1). In our hands PBMCs of AD patients in vitro stimulated by A $\beta$ 42 are able to express also higher levels of chemokines and chemokines receptors. Moreover the contribution of immunological factor in the ethiopathogenesis of AD is increasingly noted, indeed peripheral changes of the immune system such as lymphocytes function and subset distribution have been recently reported. In order to investigate about the modifications of the immune system in elderly people who might be genetically advantaged for longevity, we have studied some immunological parameters in a group of Centenarian Offspring (CO) comparing the results to data obtained in Age-matched healthy controls (AM) and young controls (YC). Our data show that CO have a more “young” immune system when compared to AM and YC.

Our works add data on the immune-inflammatory environment of Alzheimer's disease. Moreover results here shown support the hypothesis of a “familiar youth” of the immune system that can be a big advantage both to fight the main age-related diseases and to properly respond to vaccinations.

These data might be used as Biomarkers of ageing to predict the onset of age-related diseases, to identify individuals at high risk of developing diseases or disabilities and/or residual lifetime, for monitoring the effectiveness of therapeutic interventions.

*Session IV: Novel therapeutic targets and strategies in human disease*

## **Non-transcriptional control of DNA replication by c-myc: novel mechanism for its oncogenic function**

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The c-Myc proto-oncogene encodes a transcription factor that is essential for cell growth and proliferation and is broadly implicated in tumorigenesis. However, the biological functions required by c-Myc to induce oncogenesis remain elusive.

In order to identify new partners of c-Myc, we isolate the c-Myc containing protein complex from cell using an epitope-tagging strategy. Among the proteins that have been identified by mass-spectrometry analysis we focus our attention to Mcm7, Mcm5. These two proteins belong to a complex of six proteins (Mcm2-7). It has been demonstrated that the MCM complex plays a key role in DNA replication. We characterize the interaction between c-Myc and Mcm7 and demonstrate that c-Myc actually steadily interacts with the entire MCM complex along the cell cycle. Depletion of c-Myc from Xenopus cell-free extracts, which are devoid of RNA transcription, demonstrates a non-transcriptional role for c-Myc in the initiation of DNA replication. Overexpression of c-Myc causes increased replication origin activity with subsequent DNA damage and checkpoint activation. We show that c-Myc has a direct role in the control of DNA replication. These findings identify a critical function of c-Myc in DNA replication and suggest a novel mechanism for its normal and oncogenic functions.

## **Genome-wide analysis of estrogen signaling in hormone-responsive human breast cancer cells**

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Estrogen hormones promote breast cancer growth and progression by controlling cell proliferation and key cellular functions *via* the estrogen receptors (ERs). Two estrogen receptor subtypes, ER $\alpha$  and ER $\beta$ , are known to mediate estrogen signaling by functioning as ligand-dependent transcription factors and drive gene cascades comprising primary genes, whose transcription is directly controlled by the hormone through physical interaction of ERs with regulatory sites in the genome (genomic pathway) and/or with signal transduction effectors (non genomic pathway), as well as downstream genes whose activity depends upon the functions encoded by the primary responders. Expression of the two ERs shows a strikingly different pattern in breast cancer, with higher ER $\alpha$  and lower ER $\beta$  levels observed in malignant cells compared to normal mammary epithelial or benign tumor cells. This suggests a likely oncosuppressor function of ER $\beta$ .

To identify gene pathways underscoring the genomic actions of both ER subtypes in breast cancer cells, we performed a comprehensive analysis of ER $\alpha$  and ER $\beta$  target sites in the genome by integrating time-course mRNA and miRNA expression profiling following 17 $\beta$ -estradiol (E2) treatment of hormone-starved cells with global mapping of *in vivo* receptor binding sites in the genome by chromatin IP coupled to massively parallel sequencing (ChIP-Seq) and *in silico* analyses of the transcription units and receptor binding regions identified.

These results obtained provide a first comprehensive view of the dynamic gene networks controlled by estrogen *via* ER $\alpha$  and ER $\beta$  in breast cancer cells and evidence for the involvement of a number of TFs and miRNAs in estrogen mediated regulation of breast cancer cell functions, identifying key genetic determinants of the hormone-responsive tumor cell phenotype.

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## Natural lipids and antimycobacterial activity

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Tuberculosis (TB) is a worldwide infection disease, due to a single pathogen infection, which is estimated to kill over 2 million people annually. The causative agent of TB is *Mycobacterium tuberculosis* (MTB), an intracellular pathogen which infects human host and interferes with host antimicrobial pathways to allow intracellular survival, inhibiting Phospholipase D (PLD) dependent Phosphatidic Acid (PA) generation. The present study shows a novel role of synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides (CpG ODN), Lysophosphatidic Acid (LPA) and Sphingosine 1-phosphate (S1P) in the activation of antimicrobial immune response by innate immune cells, such as human monocytes/macrophages and type II human alveolar epithelial cells alveolar. In this context, we found that CpG, LPA and S1P stimulation i) induce  $\text{Ca}^{++}$ -dependent PLD activation, ii) promote PLD-dependent phagolysosome maturation, and iii) enhance a PLD dependent intracellular mycobacterial killing. These results show that alveolar epithelial cells may represent efficient effector cells of innate antimycobacterial immune response and that PLD activation i) is crucial for the activation of phagolysosome maturation-based antimicrobial response, ii) is conserved among cell types and iii) is at the intersection of different metabolic pathways, independent of the upstream stimulus received. In order to deliver these second lipid messengers able to recover or enhance host antimicrobial innate immune response to infected cells, a technological platform has been developed. In this context, apoptotic body-like liposomes characterized by the presence of phosphatidylserine (PS) in the outer lipid leaflet were engineered to contain PA in the inner lipid surface. We demonstrate that these liposomes can be internalized by macrophages and reduce production of proinflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-12, IL-23) by simultaneously inducing the production of IL-27. Moreover, the stimulation of human macrophages with apoptotic body like liposomes lead to  $\text{Ca}^{++}$  mobilization,  $\text{Ca}^{++}$ -dependent phagolysosome maturation, and phagolysosome maturation dependent intracellular mycobacterial killing. Altogether, these results suggest the possibility to use apoptotic-like vesicles as Trojan particles to deliver second lipid messengers to restore those molecular pathways inhibited by intracellular pathogens as an intracellular survival strategy.

## **Antiangiogenic activities of chemopreventive drugs**

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Angiogenesis is a limiting step for the switch from a dormant to a malignant state in tumor progression. During tumor development, tumor cells induce profound changes in the surrounding tissue, leading to the formation of an altered tumor microenvironment that is critical in permitting further tumor growth and tumor progression. We noted that angiogenesis, particularly 'inflammatory angiogenesis', is a common target of many chemopreventive molecules where they most likely suppress the angiogenic switch in pre-malignant tumors, a concept we termed "Angioprevention". Chemoprevention focuses on the primary or secondary prevention of cancer using natural or synthetic agents usually showing mild or no collateral effects.

We evaluated *in vitro* and *in vivo* the antiangiogenic activity of two synthetic oleanoleic acid-derived triterpenoids under study for their chemopreventive and therapeutic potential, CDDO -Me and -Im. *In vitro*, both compounds exhibited a strong inhibitory activity on endothelial cells isolated from umbilical veins (HUVEC). Further, these compounds inhibited chemotaxis of inflammatory cells towards specific chemokine stimuli. The triterpenoids interfered with activation of the PI3K/Akt pathway following VEGF stimulation as well as activation of the NF-kappaB pathway subsequent to TNFalpha stimulation. Moreover, CDDO-Me induces cell death in androgen-responsive and unresponsive human prostate cancer cell lines at very low concentrations, by acting against GS3K pathway (Venè R, Larghero P, Arena G, Sporn MB, Albini A, Tosetti F. *Cancer Res.* 2008). *In vivo* we performed the matrigel sponge assay; on day +4 from matrigel injection, CDDO-treated mice showed reduction of both CD31<sup>+</sup> cell number and Hb content in the sponges. These data confirm the antiangiogenic and anti-inflammatory activity of this triterpenoid, suggesting its use to develop new compounds to be employed in blocking tumor progression.

Another promising chemopreventive agent acting with different mechanism is the rexinoid LG100268. In the light of these results, an *in vivo* experiment was performed to test the efficacy of the triterpenoid CDDO-Me and the rexinoid LG100268 together in equimolar concentrations mixed with matrigel before s.c. injection. This combination succeeded in preventing matrigel vascularization *in vivo* and was active also *in vitro*.

Our data confirm the anti-angiogenic and anti-inflammatory activity of triterpenoids, and supports the idea that combination of chemopreventive inhibitors could be more effective than a mono-treatment.

Another compound interesting is "Hyperforin", the major lipophilic compound contained in extracts of Hypericum perforatum, responsible for the antidepressant activity associated with the extract. Hyperforin has been found to inhibit tumour invasion and angiogenesis. We showed that treatment with Hyperforin restrained, in a dose-dependent manner, the capacity of endothelial cells to migrate towards relevant chemotactic stimuli. Hyperforin inhibited the morphogenesis of HUVECs, and potently repressed *in vivo* in the Matrigel sponge assay in response to diverse angiogenic agents. Immunofluorescent staining showed that in cytokine-activated endothelial HUVE cells Hyperforin prevents translocation to the nucleus of NF-κB, a transcription factor

regulating numerous genes involved in cell growth, survival, angiogenesis and invasion. Under Hyperforin treatment *in vivo*, the growth of Kaposi's sarcoma – a highly angiogenic tumour – is strongly inhibited, with the resultant tumours remarkably reduced in size and in vascularization as compared with controls. Hyperforin has also been reported to have anti-inflammatory properties.

We also showed that Hyperforin inhibited neutrophil and monocyte chemotaxis *in vitro* and angiogenesis *in vivo* induced by angiogenic chemokines (CXCL8 or CCL2). These results highlight the potential for Hyperforin as an anti-inflammatory angioprevention agent, acting as a strong inhibitor of inflammation- or tumour-triggered angiogenesis, and provide new therapeutic approaches for halting pathology-associated angiogenesis.

## **Urokinase plasminogen activator receptor signalling in tumor growth and metastasis**

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The scenario of mesenchymal-type (protease-dependent) cell invasion has changed in 1985, following the first description of the receptor for urokinase-type plasminogen activator (uPAR, CD87) (1-3). It became evident that cell invasion is a surface-driven event that develops according to a well defined sequence: binding of urokinase-type plasminogen activator (uPA) to uPAR, outward exposure of uPA catalytic site, activation of plasminogen (PG) to plasmin (PL), PL-dependent degradation of non collagen components of ECM and PL-dependent activation of pro-matrix metallo proteases (pro-MMPs) to active MMPs and degradation of ECM collagens. We have later shown that uPA stimulated chemotactic and chemo-invasive migration of uPAR expressing cells by an enzymatic activity-independent mechanism, only relying on the interaction of receptor-ligand specific sequences (4). Observations indicating that uPAR is involved also in modulation of cell adhesive interactions with ECM molecules (such as vitronectin, VN) and with cell surface integrins, led us to propose the uPAR-dependent “grip-and-go” model of cell invasion (5). Thus, we focused on methods able to blunt uPAR expression in order to control cell invasion. We designed antisense oligonucleotides (aODN) able to suppress uPAR expression in cancer cells and to revert their invasive phenotype (6). Such anti-uPAR aODN were patented and were shown to be efficient *in vivo* in the control of experimental lung metastasis of human melanoma (7) and of bone metastasis of human prostate cancer cells (8) in nude mice. We have also studied the uPA/uPAR system of endothelial cells of patients affected by diffuse Systemic Sclerosis (SSc), a chronic disease characterized by accumulation of connective tissue in the skin and internal organs and by loss of vasculature, dependent on defective angiogenesis. Vessels of SSc patients undergo lumen narrowing, which results into hypoxia of down-stream endothelial cells and tissues. Despite hypoxia and the resulting hyper-production of angiogenic factors (FGF2, VEGF), SSc microvascular endothelial cells (SSc-MVEC) are unable to enter an angiogenic program. Microarray analysis of SSc-MVEC and of their normal counterpart (N-MVEC) has shown that SSc-MVEC hyper-express pro-angiogenic factors, whose activity becomes ineffectual for the prevailing presence of anti-angiogenic molecules (9, 10). We have experimentally addressed two of these molecules: the matrix-metallo protease 12 (MMP12) and pentraxin 3 (PTX3). MMP12 is a protease which cleaves uPAR domain 1, thus eliminating the possibility of uPA binding, as well as uPAR-cortical actin coupling, which provides the driving force for MVEC movement required in the angiogenic process (11, 12). Also inhibition of PTX3, a FGF2 inhibitor which is over-expressed by SSc-MVEC, results in a partial recovery of SSc-MVEC capillary forming activity. Taking advantage of the “lesson” of SSc, we have exploited the anti angiogenic activity of MMP12 and PTX3 to blunt tumor angiogenesis. Grafting in nude mice of human mammary tumor cells induced to over-express MMP12 and PTX3 by stable transfection resulted into inhibition of tumor development. Such inhibition was dependent on

impairment of angiogenesis and was paralleled by extensive necrosis of the tumor mass (13).

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## **Novel pathways and pharmacological tools in cellular autophagy: implications for mitochondrial quality control**

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A tuned mitochondrial function is essential for cellular homeostasis. Mitochondria buffer and decode  $\text{Ca}^{2+}$  signals, determine apoptotic response and produce the majority of bulk ATP. All this renders the quality of mitochondria a key parameter in cellular health.

There is now great interest to dissect the mechanisms responsible for mitochondrial quality in mammals and targeted autophagy (or mitophagy) emerges as the most prominent. And we have recently proposed a role for the  $\text{F}_1\text{F}_o$ -ATPsynthase endogenous regulator, IF<sub>1</sub>, in this process.

In our attempt to characterize the cell physiology of the protein we discovered that both mitochondrial structure and function are contributed by IF<sub>1</sub> since its expression defines the number of inner membrane cristae and the very coupling of the  $\text{F}_1\text{F}_o$ -ATPsynthase<sup>1</sup>. The whole volume fraction of mitochondrial web is also modulated by IF<sub>1</sub> as basal autophagosomal events increase upon silencing of IF<sub>1</sub> and are diminished in IF<sub>1</sub> overexpressing cells. We linked this to modifications in the rate of Reactive Oxygen Species (ROS) generation and proposed that IF<sub>1</sub> by impinging on fundamental aspects of mitochondrial biology may dictate the remodeling of mitochondrial reticulum<sup>2</sup>. IF<sub>1</sub> may be therefore critical in physio-pathological conditions associated to dysfunctional mitochondria such as neoplastic degeneration and metabolic diseases due to mitochondrial DNA mutations. Hence, IF<sub>1</sub> contribution in cells from patients carrying mutated mtDNA was exploited aiming to clarify how  $\text{F}_1\text{F}_o$ -ATPsynthase/IF<sub>1</sub> ratio of expression correlates to the rate of autophagosomes formation and mitochondria turnover.

We also exploited the role of the 1-(2-Chlorophenyl-N-methylpropyl)-3-isoquinolinecarboxamide (PK11195) in models of  $\text{Ca}^{2+}$  dependent autophagy searching for pharmacological compounds which may act on the efficiency of autophagic process. PK11195 modifies  $\text{Ca}^{2+}$  homeostasis via an effect on the oncogene Bcl-2<sup>3</sup> and we demonstrate that it also impinges on the onset of autophagy when this occurs in  $\text{Ca}^{2+}$  dependent fashion (e.g. via Vitamin D3). By these means, we propose that PK11195 may be a useful pharmacological tool to improve the efficiency and selectivity of cellular self regulated processes for the degradation and removal of protein aggregates and damaged organelles such as mitochondria in various pathologies. By these means, novel biological read outs to assess cellular health and tools for molecular therapies could be envisaged.

## **Is leptin signaling a new therapeutic target for breast cancer?**

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Leptin is a multifunctional hormone produced mainly by the adipose tissue and involved in the regulation of food intake and energy balance. In addition, leptin can stimulate mitogenic and angiogenic processes in peripheral organs. Because leptin levels are elevated in obese individuals and excess of body weight has been shown to increase breast cancer risk in postmenopausal women, attempts have been made to evaluate whether leptin can promote mammary tumorigenesis.

Our data suggest that the leptin system is involved in the pathogenesis and progression of estrogen dependent carcinoma. In fact, leptin is a potent modulator of Estrogen Receptor (ER- $\alpha$ ). First, it stimulates aromatase expression and, as a consequence, elevates estrogen levels via increased aromatization of androgen. Next, it can transactivate ER- $\alpha$  in a ligand-independent manner. Moreover, leptin treatment significantly potentiates the Estradiol-stimulated growth of tumor mass via an increase of E-cadherin expression. Finally, it is able to attenuate the effects of the antiestrogen ICI 182,780, therefore might be involved in the resistance of breast cancer cells to anti-estrogen therapies.

The relevance of leptin signaling in breast tumorigenesis is reinforced by the fact that the leptin/ObR system is significantly overexpressed in breast cancer biopsies, while is absent or express at very low levels in normal mammary epithelium, which suggests that leptin can influence breast cancer cells not only by endocrine and/or paracrine actions but also through autocrine pathways. Because the link existing between overexpression of leptin and ObR mRNA and obesity related stimuli, such as hyperinsulinemia or hypoxia, we investigated the molecular mechanisms underlying this phenomenon. We found more than one mechanisms of leptin overexpression in breast cancer tumors. For instance, Insulin is a major stimulant of leptin in ER-negative MDA-MB 231 cells, where increases the loading of the transcription factors HIF-1 $\alpha$  (Hypoxia inducible Factor 1 $\alpha$ ) and Sp1 to the leptin promoter, compared with less aggressive ER-positive MCF-7 cells. However, in MCF-7 cells the action of Insulin on the leptin promoter not involves SP1 but requires only the complex HIF-1 $\alpha$ /p300 and the highest HIF-1 $\alpha$  binding on the leptin promoter, was observed under physiological hypoxia.

All these findings, along with the important role of leptin in the angiogenic process, strongly suggest that leptin is truly involved in breast cancer development and progression. Therefore the efficacy of leptin targeting drugs in breast cancer management should be explored.

## **Gene therapy of cystic fibrosis**

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Cystic Fibrosis (CF) is a genetic fatal disease caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene on chromosome 7. CFTR is a chloride channel expressed on the apical side of airway epithelial cells, whose dysfunction causes either *per se* or for the hyperactivation of another channel called ENaC (sodium epithelial channel) a depletion of the periciliary liquid layer and a formation of viscous mucus layer. This limits the muco-ciliary clearance and allows the colonization of airways by opportunistic pathogens, leading to chronic inflammation and eventually to a lung destruction. Current therapies for CF are aimed at controlling the respiratory symptoms by antibiotic and anti-inflammatory treatments. Gene therapy, i.e. the transfer of a wild-type CFTR gene to the target airway epithelial cells, could be a resolute intervention. Clinical trials have demonstrated that, both viral (adenovirus and adenoassociated virus) and non-viral (liposomes) vectors are endowed with low efficacy, and cannot sustain a prolonged expression of CFTR gene because of their immunogenicity. Lentiviral (LV) vectors show interesting properties for CF gene therapy, since they integrate into the host genome and allow long-lasting gene expression. It has been demonstrated that they can transduce the airway epithelium correcting the basic electrophysiological defect in CF mice for the whole life of the animal and their repeated administration to the lung do not elicit a strong inflammatory response. Our recent data corroborate these findings, since a last-generation LV vector did not elicit a potent inflammatory response but only interferon-gamma production in transduced airway epithelial cells. Moreover, our studies are focussed on the entry mechanism of LV vectors into airway epithelial cells and on methods, such as magnetofection, to enhance this interaction in order to increase transduction efficiency in the absence of cytotoxicity. Finally, the use of LV vectors for CF gene therapy is presently being evaluated by us as a new tool for expressing small interfering RNAs in order to downregulate ENaC expression. *In vitro* results show that a LV vector carrying a silencing cassette against the  $\alpha$  subunit of human ENaC downregulated ENaC mRNA and protein expression and significantly reduced ENaC function. For these features, LV hold promise as an useful gene transfer vector in CF.