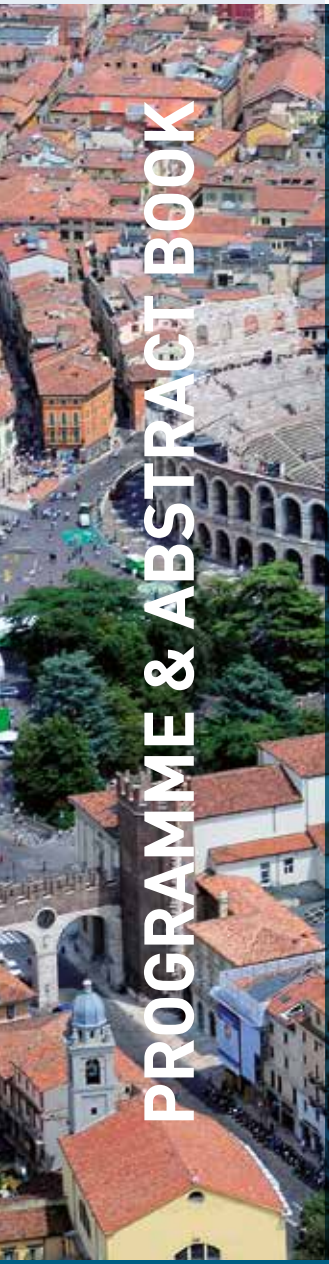
ASSOCIAZIONE ITALIANA
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UNIVERSITÀ DEGLI STUDI DI VERONA
Dipartimento Scienze della Vita e della Riproduzione
Sezione di Chimica Biologica



PROGRAMME & ABSTRACT BOOK

# 27th Annual Conference of Italian Association of Cell Cultures (ONLUS-AICC)

## OXIDATIVE STRESS AND CELL DEATH: IMPLICATIONS IN CHRONIC-DEGENERATIVE PROCESSES AND CANCER

November 12th-13th, 2014

## 5th International Satellite Symposium AICC-GISM

### ADVANCES IN MESENCHYMAL STEM CELL RESEARCH

November 14th, 2014

Auditorium Banco Popolare
Viale delle Nazioni, 4 - Verona

27th Annual Conference

## ACKNOWLEDGEMENTS

For the contribution of a generous unrestricted grant

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## November 12th, 2014 (Wednesday)

## 27th Annual Conference of Italian Association of Cell Cultures (ONLUS-AICC)

14.00 – 19.00 Registration/Information Desk Open

14.00 Poster positioning

14.30 Greetings of Authorities

15.00 **OPENING LECTURE**

**InterNET (Neutrophil Extracellular Trap) death connects autoimmunity and cancer** IS03
*Mario Colombo, Milano*

15.45 *Coffee Break*

**SESSION 1**

**Autophagy and apoptosis in cell survival control**
*Chairpersons: Massimo Donadelli, Verona - Stefania Meschini, Roma*

16.15 **Pharmacological interaction of everolimus and chloroquine in the modulation of autophagy/apoptosis in endothelial progenitor cells** IS06
*Anna Grimaldi, Napoli*

16.35 **Dissecting the role of Mitofilin and OPA1 in the cristae remodelling event and apoptosis** IS11
*Maria Eugenia Soriano, Padova*

16.55 **Synergistic effect of the HDAC inhibitor SAHA and the sesquiterpene lactone parthenolide in triple negative breast cancer cells** IS04
*Antonella D'Anneo, Palermo*

17.15 **FREE COMMUNICATIONS**

**Zn(II)-compound degrades mutant p53 through autophagy** FC04 | P20
*Alessia Garufi, Roma*

17.25 **cAMP-dependent protein kinase controls a metabolic switch essential for cancer cell resistance to glucose deprivation** FC08 | P36
*Roberta Palorini, Milano*

17.35 **Chronic myeloid leukaemia-derived exosomes promote tumour growth through an autocrine mechanism** FC09 | P41
*Stefania Raimondo, Palermo*

17.45 **Triggering of CD99 by 0662 mAb induced endocytosis of Ras and cell death in Ewing sarcoma** FC10 | P44
*Mario Terracciano, Bologna*

17.55 General discussion

18.05 AICC Members Meeting

**IS** *Invited Speakers*
**FC** *Free Communications*
**P** *Posters*

# Scientific Programme

## November 13th, 2014 (Thursday)

08.30 – 18.30 Registration/Information Desk Open

**SESSION 2**
**Reactive oxygen species: friend or foe?**
*Chairpersons: Sonia Emanuele, Palermo - Marta Palmieri, Verona*

9.00 **Stress tumorigenesis and angiogenesis: our experience** IS01
*Antonio Barbieri, Napoli*

9.20 **Homocysteine, $H_2S$ and endothelial dysfunction: role of inflammation and metalloproteases** IS07
*Diego Ingrosso, Napoli*

9.40 **ROS as cospirators for cancer malignancy: implication for cell motility, stemness and metabolic deregulation** IS02
*Paola Chiarugi, Firenze*

10.00 FREE COMMUNICATIONS
**Characterization of cell damage and death response in human cybrid models of Parkinson disease** FC01 | P03
*Davide Basello, Pavia*

10.10 **Doxorubicin metronomic chemotherapy overcome drug resistance by targeting cancer cell mitochondrial metabolism** FC06 | P24
*Joanna Kopecka, Torino*

10.20 **The TrkAIII oncoprotein inhibits mitochondrial free radical ROS-induced death of SH-SY5Y Neuroblastoma cells by augmenting SOD2 expression and activity at the mitochondria, within the context of a tumour stem cell-like phenotype** FC07 | P26
*Andrew Mackay, L'Aquila*

10.30 General discussion

10.40 *Coffee Break*

**SESSION 3**
**Role of microRNAs in cell proliferation and survival**
*Chairpersons: Paola Chiarugi, Firenze - Francesca Zazzeroni, L'Aquila*

11.10 **miRNA therapeutics a new frontier for the experimental treatment of multiple myeloma** IS13
*Pierfrancesco Tassone, Catanzaro*

11.30 **Gain-of-function activities of natural mutants of p53 in human cancers: role in gene and microRNA transcriptional deregulation** IS05
*Silvia Di Agostino, Roma*

11.50 **Role of miR34a as indicator of prognosis in sarcomas** IS10
*Katia Scotlandi, Bologna*

12.10 FREE COMMUNICATION
**Locked Nucleic Acid (LNA)-miR-221 Inhibitor as promising new anti-myeloma agent** FC03 | P17
*Maria Teresa Di Martino, Catanzaro*

12.20 General discussion

## November 13th, 2014 (Thursday)

12.30 **Seahorse Bioscience - Simplifying Cellular Bioenergetics** *non–CME certified*
*Alex Liversage*

12.45 **Application of microarray in oncology research** *non–CME certified*
*Gennaro De Vita*

13.00 **Importanza dell'acqua ultrapura nella ricerca scientifica e nelle tecniche di analisi-impatto della gestione e dello stoccaggio nella purezza dell'acqua da laboratorio** *non–CME certified*
*Antonio Di Bartolo*

13.15 AICC 2014 Awards Ceremony

13.30 *Lunch and Poster session*

**SESSION 4**
**Cancer stem cells and targeted-therapy**
*Chairpersons: Michele Caraglia, Napoli - Aldo Scarpa, Verona*

15.00 **Next generation histopathology and cancer stem cells** **IS08**
*Aldo Scarpa, Verona*

15.20 **Cancer integromics: a physician opinion** **IS12**
*Pierosandro Tagliaferri, Catanzaro*

15.40 **G-quadruplex DNA structure as target for antitumoral therapy** **IS15**
*Pasquale Zizza, Roma*

16.00 **The evolving concept of cancer stem cells, a tangled topic in the molecular biology and clinic of cancer** **IS09**
*Roberto Scatena, Roma*

16.20 **Free communications**
**Pancreatic cancer stem cells give rise to vasculogenic mimicry and secrete potential diagnostic markers** **FC02 | P15**
*Dando Ilaria, Verona*

16.30 **CXCR4 cyclic peptide antagonist (PepR) – conjugated liposomes (PL–PepR): efficacy and specificity improvement** **FC05 | P42**
*Stefania Scala, Napoli*

16.40 General discussion

16.50 *Coffee Break*

17.20 **MAIN LECTURE**
**Evolution of target therapy and personalized cancer therapy** **IS14**
*Giampaolo Tortora, Verona*

17.40 Presentation of the 5th International Satellite Symposium AICC-GISM

## November 14th, 2014 (Friday)

## 5th International Satellite Symposium AICC-GISM
## ADVANCES IN MESENCHYMAL STEM CELL RESEARCH

08.30 – 17.30 Registration/Information Desk Open

9.00 **OPENING LECTURE**
**Mesenchymal Stromal Cells: from biology to standardisation** IS25
*Mauro Krampera, Verona*

**SESSION 1**
**Cardiovascular diseases**
*Chairpersons: Giulio Alessandri, Milano - Maria Luisa Torre, Pavia*

9.30 **Mesenchymal Stem Cells: paracrine mechanisms in heart repair** IS23
*Massimiliano Gnecchi, Pavia*

9.50 **Mechanisms of Cardiac Stem Cell senescence in heart failure** IS17
*Antonio Beltrami, Udine*

10.10 **Bone marrow-derived mesenchymal Stem Cells reduce arteriotomy-induced stenosis in rat carotids** IS22
*Umberto Galderisi, Napoli*

10.30 General discussion

10.40 **Free communications**
**Mesenchymal Stem Cells from vertebral body and iliac crest bone marrow: comparison of two tissues and two harvesting techniques** FC12 | P58
*M. Evangelos Fragkakis, Leeds, UK*

10.50 **Characterization of tendon stem/progenitor cells and in vitro comparison with adipose derived stem cells: a new source for regenerative medicine?** FC15 | P66
*Deborah Stanco, Milano*

11.00 *Coffee Break*

**SESSION 2**
**Neurological diseases**
*Chairpersons: Maura Ferrari, Brescia - Enrico Lucarelli, Bologna*

11.30 **Mesenchymal Stem Cells and Parkinsonism** IS26
*Rosaria Giordano, Milano*

11.50 **Mesenchymal Stem Cells nanovesicles: innovative therapeutic approach for neuroprotection and neuroregeneration** IS18
*Bruno Bonetti, Verona*

12.10 **Mesenchymal Stem Cells in amyotrophic lateral sclerosis** IS21
*Katia Mareschi, Torino*

12.30 General discussion

# Scientific Programme

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**Role of WNT/beta-catenin up-regulation, cadherins alterations and p-runx2 expression in fibro-osseous lesions of the jaws A tissue microarray study** FC11 | P57
*Marina Di Domenico, Napoli*

12.50 **Efficacy of Mesenchymal Stromal Cells therapy for the treatment of idiopathic autoimmune inflammatory diseases of the canine central nervous system** FC13 | P69
*Zeira Offer, Lodi*

13.00 *Lunch*

14.00 **MAIN LECTURE**
**MSCs in bone and cartilage repair** IS24
*Elena Jones, Leeds, UK*

**SESSION 3**
**Mesenchymal Stromal Cells reprogramming**
*Chairpersons: Laura De Girolamo, Milano - Augusto Pessina, Milano*

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*Domenico Coviello, Genova*

14.50 **A chemical approach to cell reprogramming** IS16
*Luigi Anastasia, Milano*

15.10 **Induced pluripotent stem cell for studying development and diseases** IS20
*Nicola Elvassore, Padova*

15.30 **FREE COMMUNICATIONS**
**Cell proliferation in 3D cancer spheroids: Volume assessment and 3D reconstruction from a single 2D projection** FC14 | P64
*Filippo Piccinini, Bologna*

15.40 **Web-BI – ICT for stem cell research** FC16 | P67
*Lorenzo Vandoni, Verbania*

15.50 General discussion

16.00 Poster Awards

16.20 Closing remarks

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# NOTES

# ABSTRACTS

## Stress tumorigenesis and angiogenesis: our experience

Barbieri Antonio[1], Bimonte Sabrina[1], Palma Giuseppe[1], Luciano Antonio[1], Rea Domenica[1], Giudice Aldo[1], Scognamiglio Giosuè[2], La Mantia Elvira[2], Franco Renato[2], Zappavigna Silvia[3], Stiuso Paola[3], Caraglia Michele[3], Arra Claudio[1]

[1]Animal Facility Unit, National Institute of Tumours "Pascale", Naples, Italy
[2]Pathology Unit, National Institute of Tumours "Pascale", Naples, Italy
[3]Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, Italy

Accumulating evidence suggests that chronic stress can be a cofactor for the initiation and progression of cancer. Here we evaluated the role of endothelial nitric oxide synthase (eNOS) in stress-promoted tumour growth of murine B16F10 melanoma cell line in C57BL/6mice. Animals subjected to restraint stress showed increased levels adrenocorticotropic hormone, enlarged adrenal glands, reduced thymusweight and a 3.61-fold increase in tumour growth in respect to no-stressed animals. Tumour growth was significantly reduced in mice
treated with the β-antagonist propranolol. Tumour samples obtained from stressed mice displayed high levels of vascular endothelialgrowth factor (VEGF) protein in immunohistochemistry. Because VEGF can induce eNOS increase, and nitric oxide is a relevant factor in angiogenesis, we assessed the levels of eNOS protein by Western blot analysis. We found a significant increase in eNOS levels in tumour samples from stressed mice, indicating an involvement of this enzyme in stress-induced tumour growth. Accordingly, chronic stress did not promote tumour growth in eNOS_/_ mice. These results disclose for the first time a pivotal role for eNOS in chronic stress-induced initiation and promotion of tumour growth. Furthermore we decided to examine its possible role in the development and metastasis formation, by in vitro and in vivo studies. We demonstrated that treatments with norepinephrine (Ð2 -adrenoreceptor agonist) in mice injected with human prostate cancer cells DU145 increased the metastatic potential of these cells. Specifically, we showed that the treatment of mice with norepinephrine leads to a significant increase of the migratory activity of cancer cells in a concentration-dependent manner and that this process is blocked by propanolol (Ð-adrenergic antagonist). Mice treated with norepinephrine, displayed an increased number of metastatic foci of DU145 cells in inguinal lymph nodes and also showed an increased expression of MMP2 and MMP9 in tumor samples compared to controls. Moreover we demonstrated that the propanolol induced in norepinephrine treated DU145 cells a E-cadherin finger-like protrusions membrane drived by vimentin remodeling.

# ROS as cospirators for cancer malignancy: implication for cell motility, stemness and metabolic deregulation

Chiarugi Paola

Department Experimental and Clinical Biomedical Sciences, Biochemistry
Human Health Medical School, University of Florence, Italy

Recent advances on the role of Reactive Oxygen Species (ROS) as signaling intermediates downstream of activated growth factor receptors and adhesion molecules have provided a biochemical framework to understand how intracellular redox changes can impact on cellular functions relevant to the metastatic process like proliferation, motility/adhesion, survival.
While much experimental evidence point to a key role for ROS in carcinogenesis, the intimate connection linking intracellular redox imbalance with cellular metabolism and with cell response to microenvironmental determinants like hypoxia and inflammation is emerging as particularly critical for the establishment of tumor metastatic capacity. Accordingly, alongside with the sequence of events leading to a prototypical carcinoma metastasis (i.e. epithelial-to-mesenchymal transition (EMT), anchorage-independent growth, survival in the bloodstream, and ectopic growth), cells need to adapt to oxidative stress and exploit the redox-based intracellular signals recur as the two complementary aspects of the same malignant cell strategy to cope with an hostile microenvironment. Recent studies underline the importance of cell antioxidant capacity in metastasis, as the result a complex cell program involving enhanced motility and a profound change in energy metabolism. The glycolytic switch (Warburg effect) observed in malignant tissues is triggered by activation of redox-sensitive transcription factors, and results in an increase of cell resistance to oxidants. On the other hand, cytoskeleton rearrangement underlying cell motile and tumor-escaping behavior use ROS as intermediates and are therefore facilitated by oxidative stress.
I therefore present metastasis as an integrated "escape program" triggered by redox changes and aimed at avoiding oxidative stress within the primary tumor. This novel perspective suggests that metastasis represents an integrated strategy for cancer cells to avoid oxidative damage and escape excess ROS in the primary tumor site, explaining why redox-signaling pathways are often up-regulated in malignancy and metastasis.

# InterNET (Neutrophil Extracellular Trap) death connects autoimmunity and cancer

Colombo Mario Paolo

Molecular Immunology Unit, Dept. of Experimental Oncology and Molecular Medicine
Fondazione IRCCS Istituto Nazionale dei Tumori, Milano, Italy

Neutrophils have been associated to the pathogenesis of Systemic Lupus Erythematous (SLE) and their peculiar death, called NETosis, has been documented at site of vasculitis. Anti-neutrophil cytoplasmic antibodies (ANCA) are autoantibodies targeting proteins normally retained within neutrophils. NET are decondensed chromatin threads decorated with cytoplasmic proteins endorsed with anti-microbial activity. We have shown that NET are also the source of auto-antigens triggering vasculitis. This has been demonstrated uploading and activating mDC with NET components that injected into naive mice were able to stimulate ANCA and autoimmunity. Such DC upload and DC-mediated autoimmunity were prevented by NET treatment with DNAse, indicating that NET structural integrity is necessary to maintain the antigenicity of cytoplasmic proteins.
Our knowledge of increased neutrophil accumulation and persistent immune system activation occurring in Sparc null mice and the reported SPARC down-regulation in the gene signature characterizing diffuse large B-cell lymphoma with poor prognosis, prompted the hypothesis of SPARC loss/down-regulation in the transition from autoimmunity to lymphoma. To this aim, the lymphoproliferative spur proper of fas mutant mice (lpr-lpr, BALB/c strain) has been transferred into the matricellular protein deficient Sparc-/- background. Single and double mutant mice were followed for development of autoimmunity (renal vasculitis, spleen weight and structure modification, autoantibodies) and lymphoma. The lack of SPARC in lpr-lpr/Sparc-/- mice anticipated the onset of autoimmunity and promoted early B cell lymphoma development, an event rarely observed in aged lpr-lpr/SPARC+/+ mice. We identified B1 cells as the B cell population undergoing malignant transformation in the Sparc-/- background. B1 cells, which localize vascular niches rich in collagen type IV/fibronectin and few neutrophils in the spleen of lpr/lpr mice, were instead localized in a niche almost devoid of ECM while rich in neutrophils undergoing NETosis, in the spleen of lpr-lpr /Sparc-/- mice. In this setting, B1 cells gain tumorigenic properties and form tumors when transplanted into nude mice. B1 lymphomagenesis promoted by SPARC deficiency in the lpr-lpr autoimmune setting represents a formal link between matricellular proteins, autoimmunity, and lymphoid malignancies.
The findings uncover the existence of lymphoid clones that largely rely on biased stromal signals like CD5+ B cells that find in CLL the human correlative.

# Synergistic effect of the HDAC inhibitor SAHA and the sesquiterpene lactone parthenolide in triple negative breast cancer cells

D'Anneo Antonella[1], Lauricella Marianna[2], Carlisi Daniela[2], Emanuele Sonia[2], Buttitta Giuseppina[2], Vento Renza[1], Tesoriere Giovanni[1]

[1]Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche, Università di Palermo, Italy
[2]Dipartimento di Biomedicina Sperimentale e Neuroscienze Cliniche, Università di Palermo, Italy

*Objective*
The sesquiterpene lactone Parthenolide (PN) exerts cytotoxic effects on MDA-MB231 cells, a triple-negative breast cancer (TNBC) cell line. However, PN is ineffective at low doses, thus restricting its therapeutic potential. This study shows that the association of the histone deacetylase inhibitor SAHA (suberoylanilide hydroxamic acid) to PN clearly improves the cytotoxic activity of this drug.
Materials and Methods: After a pre-treatment with 2 μM SAHA for 20h, MDA-MB231 cells were co-incubated with SAHA/PN for various times. The analyses were performed by: MTT cell viability test, monodansylcadaverine staining for autophagy, AnnexinV-PI for apoptosis, colorimetric method for GSH, JC-1 test for mitochondrial membrane potential, fluorescence microscopy for Nrf2 localization, western blotting for factors involved in PN/SAHA molecular mechanism.

*Results*
Our results demonstrated that SAHA synergistically interacts with PN lowering MDA-MB231 cell viability. PN alone stimulated Akt/mTOR survival pathway and promoted the nuclear translocation of Nrf2, while treatment with SAHA alone induced an autophagic process. However when the cells were exposed to SAHA/PN combination, SAHA suppressed PN effect on Akt/mTOR/Nrf2 axis, while PN reduced SAHA-induced prosurvival autophagy. In addition combined treatment triggered apoptosis with GSH depletion, dissipation of ΔĐm, release of cytochrome c and activation of caspase 3. We also demonstrated that SAHA/PN treatment maintained both H3 and H4 histone hyperacetylation induced by SAHA. Moreover, two important effects induced by PN, namely the down-regulation of DNMT1 expression as well as the inhibition of the DNA binding activity of NF-kB were observed also after combined treatment.

*Conclusions*
We demonstrated that a synergistic interaction occurs between SAHA and PN and ascertained the molecular nature underlying their effect showing that PN/SAHA combination inhibits the cytoprotective responses induced by the single compounds, while does not alter the mechanisms leading to the cytotoxic effects.

# Gain-of-function activities of natural mutants of p53 in human cancers: role in gene and microRNA transcriptional deregulation

Di Agostino Silvia

Translational Oncogenomic Unit, Molecular Medicine Area, Regina Elena National Cancer Institute, Rome, Italy
Molecular Chemoprevention Group, Molecular Medicine Area, Regina Elena National Cancer Institute, Rome, Italy

The TP53 tumour suppressor gene is mutated in approximately half of human cancers. Missense mutations in the TP53 gene are among the most common alterations found in human cancer cells. In vitro and in vivo evidences pointed out that GOF (Gain of Function) mutant p53 proteins promote increasing chemoresistance, invasion, metastasis and structural chromosomal changes resulting in high levels of genomic instability in tumors of different tissue origin. TP53 mutations are generally associated with aggressive cancers and poor patient prognosis.
However the exact vastness of mutant p53 contribution to cancer progression in different backgrounds and the specificity of its variants remain to be characterized.
During the last years of research the questions of our group are how mutant p53 proteins could alter the transcriptional profile and if a core mutant p53 program can be identified at transcriptional level (gene and microRNA expression).
Along this way we have previously described the interactions of mutant p53 proteins with other transcriptional factors and cofactors as p73, NF-Y, p300, E2F1 promoting an aberrant transcriptional regulation and sustaining hyperproliferation, metastatization and chemoresistance in human cancer cells.
Here we show that RAD17 and BRCA1 genes, whose derived proteins play a pivotal role in DNA damage repair, are transcriptional targets of gain-of-function p53 mutant proteins. Indeed, high levels of mutant p53 protein facilitate DNA damage accumulation and severely impair RAD17 and BRCA1 expression in proliferating cancer cells. We show that the recruitment of mutp53/E2F4 complex onto specific regions of RAD17 and BRCA1 promoters leads to the inhibition of their expression.
Furthermore, BRCA1 and RAD17 protein expression is reduced in Nead and Neck carcinoma patients (HNSCC) carrying TP53 mutations when compared to those bearing wt-p53 gene. The analysis of gene expression databases for breast cancer patients reveals that low expression of DNA repair genes correlates significantly with reduced relapse free survival of patients carrying TP53 gene mutations.
Beyond the modulation of gene expression, we recently showed that mutant p53 proteins are involved in transcriptional deregulation of microRNA expression. Specifically, mutant p53 binds the miR-223 promoter and reduces its transcriptional activity. This requires the transcriptional repressor ZEB-1. miR-223 exogenous expression sensitizes breast and colon cancer cell lines expressing mutant p53 to treatment with DNA -damaging drugs. Among the putative miR-223 targets, we focused on stathmin-1 (STMN-1), an oncoprotein to confer resistance to chemotherapeutic drugs associated with poor clinical prognosis.
The comprehension of the molecular events governed by GOF mutant p53 proteins might lead to the identification of cancer signalling pathways which can be subsequently tackled either with existing or novel anticancer therapies.

# Pharmacological interaction of everolimus and chloroquine in the modulation of autophagy/apoptosis in endothelial progenitor cells

Grimaldi Anna[1], Balestrieri Maria Luisa[1], D'Onofrio Nunzia[1], Di Domenico Gilda[2], Nocera Cosimo[2], Lamberti Monica[3], Tonini Giuseppe[4], Zoccoli Alice[4], Santini Daniele[4], Caraglia Michele[1]*, Pantano Francesco[4]

[1]Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, Naples, Italy
[2]Blood Transfusion Center ¨S. Giovanni Bosco¨ of Naples, Naples, Italy
[3]Department of Experimental Medicine, Section of Igiene, Medicina del Lavoro e Medicina Legale, Second University of Naples, Naples, Italy
[4]Campus Biomedico University, Translational Oncology PRABB, Rome, Italy

Endothelial Progenitor Cells (EPCs), a subpopulation of the mononuclear cell fraction in peripheral blood, play a critical role in cancer development as they contribute to angiogenesis-mediated pathological neovascularization. In the present study, we evaluated the effects of everolimus, a rapamycin analogue, alone or in combination with chloroquine, a 4-alkylamino substituted quinoline family member, one of the autophagy inhibitors, on EPCs biological functions. EPC isolation and characterization were performed by flow cytometry analysis; cells positive for both mouse monoclonal antibodies to human CD34-FITC and to anti-h VEGF R2/KDR-PE were judged as EPC. Apoptosis and autophagy assays were performed by flow cytometry while biochemical analysis was performed by immunoprecipitation and western blot. We found that either everolimus or chloroquine induce growth inhibition on EPCs in a dose-dependent manner after 72 h from the beginning of incubation. The combined administration of the two drugs to EPC was synergistic in inducing growth inhibition; the maximal pharmacological synergism between everolimus and chloroquine in inducing growth inhibition on EPCs cells was recorded when chloroquine was administered 24 h before everolimus. Moreover, we have studied the mechanisms of cell death induced by the two agents alone or in combination on EPCs and we have found that the synergistic effect of combination on EPC growth inhibition was paralleled by increased apoptosis induction and reduced autophagy. These effects occurred together with biochemical features that are typical of reduced autophagic death such as increased co-immunoprecipitation between Beclin 1 and Bcl-2. Chloroquine antagonized the inhibition of the activity of Akt→4EBP1 axis mediated by everolimus and at the same time it blocked the feed-back activation of Erk-1/2 induced by RAD in EPCs. These data suggest a new strategy in order to block angiogenesis in tumours in which this process plays a key role in both the sustainment and spreading of cancer cells.

# Homocysteine, $H_2S$ and endothelial dysfunction: role of inflammation and metalloproteases

Ingrosso Diego[1], Perna Alessandra F.[2]

[1]Dept of Biochemistry, Biophysics & General Pathology, S.U.N. School of Medicine & Surgery, Naples, Italy
[2]Dept of Cardiothoracic & Respiratory Sciences, S.U.N. School of Medicine & Surgery, Naples, Italy

Sulfur amino acid metabolism is characterized by strict interconnections among the methionine-homocysteine and folate cycles, as well as the trassulfuration pathway. Particularly two compounds, which are strictly related to this metabolism, have been involved as effectors on the vasculature: homocysteine and hydrogen sulfide (H2S). Hyperhomocysteinemia is a powerful cardiovascular risk factor, while H2S has been recently assessed as the third gaseous vasodilator, after nitric oxide (NO) and carbon monoxide (CO). The transsulfuration pathway is the metabolic link between the two compounds, in that it represents the major pathway for both homocysteine metabolism and H2S biosynthesis.

It has been shown that homocysteine toxicity on the vasculature is mainly mediated by a number of its own derivatives, acting through various mechanisms including epigenetic modifications (1) and inflammatory triggering. This latter has been extensively studied also using cell models. A number of chemokines and cytokines as well as A Disintegrin And Metalloproteinase 17 (ADAM17) have been shown to play a role (2). Conversely H2S has drawn scientists' attention because of its protective effects on the vasculature, as well as its derangement and, hence, potential involvement in the pathogenesis of hypertension and vascular damage e.g. in chronic kidney failure patients, through mechanisms also partly related to inflammation (3, 4).

In conclusion, derangements of sulfur amino acid metabolism offers new highlights in cardiovascular medicine for their potential involvement in the genesis of vascular damage by triggering inflammatory response.

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# Next generation histopathology and cancer stem cells

Scarpa Aldo

ARC-NET – Center for Applied Research on Cancer, Department of Pathology and Diagnostics
University of Verona, Italy

Cancer inter-tumor and intra-tumor heterogeneity, a well-known fact described by pathologists in the classification of tumors over the last two centuries, has finally risen to the forefront of clinical interest. Cancer genomics and transcriptomics studies have shown that tumors belonging to the same histotype display remarkable differences in their genetic assets; such inter-tumor heterogeneity is the basis of molecular subclassification with clinical impact for targeted therapeutic approaches. It has also become clear that phenotypically and genetically diverse clones of neoplastic cells may be juxtaposed within the same tumor. These clones are thought to be players in a branching clonal evolution scenario leading to the formation of metastases that are more aggressive and resistant to treatments than the primary tumor.

The histological and immunohistochemical characterization of multiple samples from the same tumor can highlight the presence of subpopulations of neoplastic cells displaying peculiar morphological and immunophenotypical features; this morpho-phenotypical analysis of intratumor heterogeneity finds its natural complement in a comprehensive characterization of molecular lesions within a cancer specimen. The sum of these data offers essential information to diagnose and subclassify cancers for the scope of determining prognosis and selecting tailored treatments.

The sequencing analysis of hotspot mutations in cancer-related genes has thus become a useful tool in selecting personalized therapy for many malignancies. However, the use of conventional techniques for a wide molecular characterization of tumors is hampered by the high costs and time needed to assess multiple molecular alterations, and by the limited amount of tissue consisting in formalin-fixed paraffin-embedded (FFPE) biopsies and/or fine needle aspiration cytology. This calls for the implementation of companion diagnostic methods for (i) simultaneously testing multiple genetic alterations and (ii) quantifying the molecular subclones, i.e. the amount of cancer cells harboring any different mutation. Massive parallel sequencing, also known as next-generation sequencing (NGS), has recently been introduced and is the most sensitive approach to index multiple genes starting from a limited amount of DNA. In the present study, we assayed a targeted multigene NGS (TM-NGS) test in 35 FFPE samples from diverse upper gastrointestinal tract tumors to define its diagnostic potential in characterizing cancer molecular heterogeneity.

A "next-generation histopathologic diagnosis" encompasses the possibity to integrate morphologic, immunophenotypic, and mutational analysis of multiple genes using routinely processed tissues. Morphology and immunohistochemistry provide the diagnosis and drive the choice of areas to be microdissected and used for multiplex deep sequencing.

This geographic mutational analysis hold the promise to let us reconsider the current WHO classifications of cancer; presents as a potent diagnostic complement to histopathologic and immunophenotypic diagnosis, able to trace the clonal evolution of the neoplasm, thus permitting the description of cancer heterogeneity in a diagnostic report; and identifies potential therapeutic targets for which agents that are currently in clinical trials for different tumor types could be of use.

The capacity to isolate cancer stem cells from primary tumors and their analysis with next generation sequencing technologies will permit a thorough evaluation of the molecular landscape of the initating cells of individual tumors. This has already been demonstrated as a proof of concept to personalize cancer therapy. The time has come to put our efforts in introducing this novel next-generation histopathological diagnosis into the clinical practice.

# The evolving concept of cancer stem cells, a tangled topic in the molecular biology and clinic of cancer

Scatena Roberto

Catholic University, Institute of Biochemistry and Clinical Biochemistry, Rome, Italy

Cancer stem cells (CSCs) or tumor-maintaining cells or stem-like cancer cells are a subpopulation of cancer cells that show some of the characteristics of stem cells to survive and adapt to ever-changing environments. These include the ability to self-renew and the capacity to produce progenitors that differentiate into other cell types. Some molecular aspects related to CSCs can significantly modify the experimental and clinical approaches to cancer and are becoming a key area of cancer biology . In fact, CSCs represent the ideal justification for a lot of intriguing and obscure aspects of cancer pathogenesis (i.e., cancer cell dormancy, chemoresistance, local and distant relapses). Considering the enormity of the clinical implications related to CSCs, a careful identification of the molecular phenotype associated to an accurate definition of their typical plasticity which appears intriguingly related to derangements in cell differentiation and metabolism, can represent a fundamental advance in terms of early diagnosis and selective therapy of cancer. At last but not least, the knowledge of pathogenetic mechanisms at the basis of CSCs can enlarge and ameliorate the therapeutic applications of the normal adult stem cells (i.e., regenerative medicine, tissue engineering, biotechnology applications) by reducing the risk of a deranged, uncontrolled, and thereby directly and/or indirectly tumorigenic potential which could be a serious drawback of regenerative medicine in general and of MSCs use in particular.

# Role of miR34a as indicator of prognosis in sarcomas

Scotlandi Katia

CRS Development of Biomolecular Therapies, Experimental Oncology Lab, Rizzoli Institute, Bologna, Italy

*Introduction:*
A large number of studies have demonstrated how detection of miRNAs expression either in tumors or more recently in blood can be used as indicator of outcome in different type of tumors. The aim of this study was to validate miR-34a expression for prognosis prediction of Ewing sarcoma (EWS) and osteosarcoma (OS) patients. Previous studies have indeed highlighted this miRNA as one of the most important in the progression of these two primary tumors of bone (Nakatani F, 2012; Bae Y, 2013).

*Material and Methods*
Localized tumors from patients with non-metastatic EWS or OS treated at the Rizzoli Institute with neo-adjuvant chemotherapy and lung metastases were studied. Expression of miR-34a and of some of its targets (cyclin D1, bcl-2, SIRT1 and YY1) was evaluated by qRT-PCR using TaqMan MicroRNA Assays and/or by immunohistochemistry on tissue microarrays from the same patients. Chemosensitity towards conventional agents (DXR, VCR) was evaluated in vitro in cells transfected with miR-34a mimics, non-specific control miRNAs (miRNC) or antagomir (100 nM) 24 h after cell seeding.

*Results*
High expression of miR-34a in localized tumors was significantly related to better event-free and overall survival in both EWS and OS. Relevance of miR-34a was confirmed by using different calibrators (normal mesenchymal stem cells and different normal tissues). By multivariate Cox regression analysis, low miR-34a expression was confirmed as independent risk factors associated with poor outcome. Expression of miR-34a was lower in metastases than in primary tumors. In clinical samples miR34a expression inversely correlated with expression of cyclin D1 and Ki-67. Functional analysis of miR-34a in EWS cell lines indicated that when miR-34a expression was enforced, cells were less proliferative, less malignant, and sensitized to doxorubicin and vincristine.

*Conclusions*
By confirming its relationship with clinical outcome, we propose evaluation of miR-34a at diagnosis of EWS and OS patients to allow early risk stratification. Restoration of miR-34a activity may be useful to increase tumour sensitivity to current drugs, so sparing excessive long-term toxicity to patients who in most of the cases are children or adolescents.

## Dissecting the role of Mitofilin and OPA1 in the cristae remodelling event and apoptosis

Soriano Maria Eugenia

University of Padova, Department of Biology, Padova, Italy

Mitochondria are main players in cell death. In particular, apoptotic mitochondria undergo ultrastructural changes that facilitate the cytochrome c mobilization from the cristae to the intermembrane space (IMS). This process is the so-called cristae remodeling event. Our lab has demonstrated that OPA1 is a key regulator of cristae remodeling by maintaining the mitochondrial cristae junctions closed. Remarkably, complexes containing OPA1 are disrupted after treatment with the proapoptotic protein cBID that leads to the release of cytochrome c to the IMS and then to the cytoplasm in the course of apoptosis. To identify new regulators of the cristae remodeling we have investigated the molecular nature of OPA1 complexes by SILAC labeling and three Dimensional Blue Native- Blue Native- SDS PAGE, followed by proteomic analysis (Liquid Chromatography/Mass Spectrometry). We have identified intriguing candidate proteins, among which Mitofilin stands out by decreasing fivefolds after cBID treatment. Our results show that Mitofilin silencing does not alter cristae junction diameter but affects the length of inner membrane and cristae junctions contact sites. However, the latter alteration do not affects cytochrome c release. Moreover, we observe an increase of the cristae width during Mitofilin silencing that provokes a defect in the respiratory chain supercomplexes formation and in respiratory efficiency, without dramatic consequence for the cell in no stress conditions. Still the underlying mitochondrial dysfunction can be unmasked after treatment with oligomycin. Remarkably, Mitofilin overexpression is not able to recover the phenotype in OPA1 deleted cells but , on the other hand, OPA1 overexpression recover the mitochondrial ultrastructure and cell death observed during Mitofilin downregulation. Our results demonstrate the role of Mitofilin in the cristae remodeling pathway downstream of OPA1.

# Cancer integromics: a physician opinion

Tagliaferri Pierosandro

Dipartimento di Medicina Sperimentale e Clinica, Università "Magna Graecia" di Catanzaro, Italy

The Omics revolution has opened a new hera for the study of human cancer. The availability of different "omics" as trascriptomics, metylomics, microRNA-omics, proteomics, metabolomics has urged the need of achieving an integrated architectural view of human tumors.
On these bases, we can define integromics (integrative genomics) as the study of molecular events of cancer at different levels in an attempt to integrate their effects in a functional or causal framework (1)
Different integromics methodologies are used for the interpretation of omics and clinical data from various solid and hematologic cancers and model systems requiring expertise in different disciplines, such as biology, medicine, mathematics, statistics and bioinformatics.
This new discipline has made possible by the availability for the scientific community of clinically annotated data sets from the International Cancer Genome Consortium (ICGC) and the Cancer Genome Atlas (TCGA).
Our group has focused its attention on the Integrated Analysis of miRNAs, transcription factors and target genes expression of hyperdiploid Multiple Myeloma (hd-MM) by a network based analysis using a PARADIGM-like algorithm. We found a specific molecular architecture of this disease subset as compared to non hd-MM (2). Our findings are in strong agreement with the hd-MM clinical presentation and identify druggable targets to be validated in proof-of-concepts studies.
Cancer integromics offer a highly valuable approach to the reclassification of human tumors as well as to the identification of novel therapeutic targets.
However, it has to be taken into account the euristic nature of integrative genomics, the need of both robust analytic tools and sound clinical validation methodology.
The "omics" revolution as well as integrative genomics and massive parallele sequencing strongly warrant a novel clinical research methodology for precision medicine, with innovative and highly flexible study design.

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(2) Di Martino MT et al Submitted 2014

# miRNA therapeutics a new frontier for the experimental treatment of multiple myeloma

Tassone Pierfrancesco

Università Magna Grecia, Catanzaro, Italy

Recently, it has emerged that the non protein-coding components have a pivotal role in regulating physiologic cellular processes and contributes to molecular alterations in pathologic conditions. microRNAs (miRNAs) are endogenous short non-coding RNAs (~22 nt) that control gene expression at the post-transcriptional level by targeting the 3'-UTR. So far, several findings have highlighted the role of miRNA in human cancer. It is now well established that some miRNAs can function as either oncogenes or tumor suppressors. Those miRNAs whose expression is increased in tumors may be considered as oncogenes (oncomiRs) that promote tumor development by inhibiting tumor suppressor genes and/or genes controlling cell differentiation or apoptosis; conversely, underexpressed miRNAs in cancers may function as tumor suppressor genes mainly by down-regulating oncogenes. The available evidence of extensive deregulation of miRNAs in human cancers provides a strong rationale for designing miRNA-based therapeutic strategies:
a) miRNA-inhibition approach: targeting miRNAs that are overexpressed and support survival and proliferation of the tumour clone (oncomiRs). In this scenario, antisense oligonucleotides as miRNA inhibitors with sequence-complementary to the endogenous miRNA are used to deplete tumor cells of tumor-promoting oncomiRs.
b) miRNA-replacement approach: it consists in restoring the function of miRNAs, that are down-regulated and/or selectively deleted, thus favoring tumor growth and progression. Restoring miRNA levels can be achieved either by using viral vectors over-expressing a specific miRNA, or by using synthetic double-stranded miRNAs, named miRNA mimics. We recently provided the first evidence that synthetic miR-34a may act as therapeutic agent in multiple myeloma1-3. Either transient expression of miR-34a synthetic mimics or lentivirus-based miR-34a-stable enforced expression triggered growth inhibition and apoptosis in MM cells in vitro. Synthetic miR-34a mimics down-regulated canonic targets BCL2, CDK6, and NOTCH1 at both the mRNA and protein level. Lentiviral vector-transduced MM xenografts with constitutive miR-34a expression showed high growth inhibition in severe combined immunodeficient (SCID) mice. The anti-MM activity of lipidic-formulated miR-34a was further shown in vivo in two different experimental settings: (i) SCID mice bearing nontransduced MM xenografts; and (ii) SCID-synth-hu mice implanted with synthetic 3-dimensional scaffolds reconstituted with human bone marrow stromal cells and then engrafted with human MM cells. Relevant tumor growth inhibition and survival improvement were observed in mice bearing TP53-mutated MM xenografts treated with miR-34a mimics in the absence of systemic toxicity. An important achievement was the evidence that miR-34a overcomes the human BMM-dependent protective effect on MM cells in vitro and in vivo. The translational relevance of our findings resides in the achievement of a proof-of-principle that systemic delivery of novel lipidic-formulated miR-34a mimics exert anti-MM activity in different clinically relevant murine models of human MM. The study provides a framework for clinical development of formulated miR-34a mimics in MM. Notably, miRNAtherapeutics initiated in 2013, a clinical development program of MRX34, a liposome-encapsulated miR-34 mimic, in patients with unresectable primary liver cancer or solid cancers with liver involvement. Our group also provided proof-of-principle that other miRNA, including miR-29b 4, miR-199a-5p 5 are effective in replacement strategies while other such as miR-216,7 or miR-221 are valuable targets for inhibitory approaches. Our data suggest that miRNA therapeutics will represent an innovative tool to fight cancer.

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## Evolution of target therapy and personalized cancer therapy

Tortora Giampaolo

Medical Oncology, University of Verona and Azienda Opedaliera Universitaria Integrata - Verona, Italy

The rapid progress in the tandem understanding of molecular processes undergoing tumor growth and dissemination and the development of new drugs and diagnostic tools is changing dramatically the way we approach cancer today. In the past few years major breakthrough are changing the survival of patients affected by different types of cancer such as renal, colon, and melanoma. An additional contribution is coming from the long waited translation of immune science into the clinics and more is expected from its integration with targeted therapy.

We are now aware that tumors exhibit extensive heterogeneity, both between and within tumors, and that heterogeneity, along with acquired mutations, contribute to inevitable occurrence of drug resistance.

A key contribution has been the definition of a (still relatively small) number of biomarkers predictive of response to treatment, which helps to stratify patients according to the molecular phenotype of their tumors.

This approach will help to define exactly which molecular lesion is present in each tumor of each patient in order to select for the right drugs only the patients who may really benefit of specific targeted agents. The tailoring of such so-called Personalized medicine, however, moves even beyond the approach of patients stratifying into treatment groups based on phenotypic biomarkers. In fact, a challenge for the near future is the translation of the huge body of information coming from the ongoing sequencing of the cancer genome by next-generation techniques into the clinics.

## G-quadruplex DNA structure as target for antitumoral therapy

Zizza Pasquale, Biroccio Annamaria

Regina Elena National Cancer Institute, Laboratory of Experimental Chemotherapy, Rome, Italy

G-quadruplexes (G4s) are non-canonical secondary structures that may form within guanine-rich nucleic acid sequences. Over the last two decades, telomeres, the terminal portion of chromosomes, have received much attention since they can fold into intramolecular G4s, leading to the synthesis of several G4 ligands, small molecules designed to recognize and stabilize the G4 structures. Interestingly, these ligands were shown to selectively exert an anti proliferative effect in both in-vitro and in-vivo cancer models, without affect normal samples. Such results pointed to G4s as possible candidates for anticancer target therapy. Other than in telomeres, G4 have been more recently found at additional regions dispersed within the human genome, including the promoter regions, the 5'-UTRs and the introns of several oncogenes, hallmark of cancer. Based on these observations, the therapeutic potential of the small-molecule ligands acting as G4 stabilizers is constantly increasing due to their ability to both induce telomere dysfunction and to form impediments to transcription and translation. Notably, G4 DNA structures are now visualized in human cells, corroborating the application of stabilizing ligands as a new class of anticancer agents. In this context, we are developing druggable small molecules possessing multi target activities and extremely limited off-target effects.

## A chemical approach to cell reprogramming

Anastasia Luigi

Laboratorio di Cellule Staminali per l'Ingegneria Tessutale
Ricerca biomedica in ambito biochimico e cardiovascolare
IRCCS Policlinico San Donato, San Donato Milanese (MI), Italy

The possibility of reprogramming cells by a chemical approach, i.e. by treating them with specifically designed de-differentiating molecules, has been auspicated by many authors. Moreover, small molecules could be critically important in the induction of stem cell differentiation toward the opportune phenotype, especially in the case of cardiac regeneration. The talk will report several examples of the use of small synthetic molecules in cell reprogramming and differentiation. Likewise, the possibility of using stem cells for therapeutic applications should undergo the same quality standards used for drug synthesis, and cell preparations should be pure and unequivocally defined. This long standing issue is crucial, as stem cell preparations are always mixed populations containing cell contaminants that do not possess the same plasticity and they cannot be induced to differentiate into the desired cell phenotype. Among possible new markers, glycosphingolipids represent a very interesting class of bioactive compounds, as they are exposed on the cell membrane, and it is known that their distribution changes dramatically during development. Furthermore, several aspects of glycosphingolipid involvement in stem cell proliferation, fate determination and differentiation will be discussed, together with the use of synthetic small molecules for stem cell reprogramming.

# Mechanisms of Cardiac Stem Cell senescence in heart failure

Beltrami Antonio

Dipartimento Ricerche Mediche e Morfologiche, Università degli Studi di Udine, Italy

*Background*

Human Cardiac Stem Cells (hCSCs) obtained, in vitro, from failing explanted hearts (E-hCSCs) differ from those isolated from healthy donors (D-hCSCs) for the increased expression of senescence markers and the reduced in vivo reparative abilities.

Since the autophagy lysosome pathway (ALP) plays a pivotal role in cellular homeostasis by controlling both cellular clearance and response to nutrients, defects on ALP may be associated to ageing and heart failure progression.

*Aims*

To monitor the efficiency of ALP in hCSCs isolated from patients with heart failure and to develop a drug-based strategy able to boost ALP activity.

*Methods and Results*

14 D-hCSCs and 20 E-hCSCs, obtained from healthy and failing human hearts, respectively, were compared in terms of cell surface immunophenotype and senescence marker expression. Although the two groups of cells shared a similar immunophenotype, E-hCSCs showed a significant enrichment in the fraction of senescent cells (p16+, ĐH2A.X+Ki67-).

Microarray analysis and Real Time PCR were performed to investigate 3 D- and 3 E-hCSCs for transcriptional profile and for expression profile of 380 microRNAs, identifying 452 genes downregulated in E-hCSCs ($p<0.05$), 16 of which are involved in ALP, in addition to 12 differently expressed miRNAs ($p<0.05$), 9 of which are able to target genes linked to ALP regulation.

Consistently, the lysosomal compartment of 7 D- and 5 E-hCSCs was monitored by FACS analysis after staining with lysotracker and acridine orange and E-hCSCs displayed lysosomes less functional than the D-HCSCs ones. Furthermore a significantly higher colocalization of lipofucsin and Galectin-3 was found in E-hCSC lysosomes, with respect to D-hCSC's ones.

Since, in the course of autophagy, lysosomal function is activated via mTORC1 suppression, we evaluated mTORC1 activity by western blot analysis. In particular we focused our attention on pS6K, Akt in parallel with the autophagic markers Atg3, Atg7, LC3II, p62. Altogether results showed that E-hCSCs were characterized by an enhanced activity of mTORC1 and an arrest in autophagic degradation. Moving from these elements we develop a three days drug treatment of E-hCSCs with 10nM Rapamycin (TORC1 inhibitor).

This pharmacologic strategy was able to reduce mTORC1 activity, to potentiate the lysosomal functionality, to improve the autophagic flux and to reduce the fraction of senescent cells.

*Conclusions*

We demonstrated that E-HCSCSs are characterized by a blunted ALP. The pharmacologic inhibition of TORC1 on one hand reactivated the pathway and on the other hand contrasted senescence offering promising perspectives to improve EHCSCS cardiac regenerative efficiency.

## Mesenchymal Stem Cells nanovesicles: innovative therapeutic approach for neuroprotection and neuroregeneration

Bonetti Bruno

Dipartimento di Neuroscienze, Policlinico G.B. Rossi, Università di Verona, Italy

The efficacy of mesenchymal adipose-stem cells (ASC) has been demonstrated in several neurodegenerative and neuroinflammatory animal models, although concerns about their safety have been raised, particularly related to their persistence in ectopical niches. For this purpose, we have tested the efficacy of nanovesicles (NV) from ASC in different experimental models of neurological diseases. We found that NV increased the cell viability of neuroblastoma cell line SH-SY5Y and of primary hippocampal neurons exposed to H2O2 by reducing the apoptotic rate. We also tested the neuroregenerative potential in the ex vivo model of cerebellar slice cultures incubated with the demyelinating agent lysophosphatidylcholine and found that NV induced a 2.2 fold increase of precursor cells by immunoblotting and a parallel 5 fold increase of MBP+ myelinated areas by immunohistochemistry as compared to control. Finally, we verified the efficacy of NV in vivo in the MOG-induced EAE model by intravenous injection at days 3, 5, 8 post-immunization. We found that NV significantly ameliorated the clinical course with marked decrease of inflammatory and demyelinating scores examined at disease peak. Notably, the proliferation in vitro of MOG-specific T cells was also significantly inhibited by NV treatment.
Taken together, these results show that ASC-derived NV exert neuroprotective and neuroregenerative effects and modulate the immune responses, potentially representing a novel therapeutic approach for neurological diseases.

# Generating disease-specific induced pluripotent Stem Cells (iPSCs) in a Biobank facility for genetic disease modelling

Coviello Domenico[1], Missaglia S.[2], Castagnetta M.[1], Pennisi E.M.[3], Baldo C.[1], Mogni M.[1], Tavian D.[2,4]

[1]Laboratory of Human Genetics, E.O. Ospedali Galliera, Genoa, Italy
[2]Laboratory of Cellular Biochemistry and Molecular Biology, CRIBENS, Catholic University of the Sacred Heart, Milan, Italy
[3]UOC Neurologia, A.C.O. San Filippo Neri, Rome, Italy
[4]Psychology Department, Catholic University of the Sacred Heart, Milan, Italy

The "Galliera Genetic Bank" (GGB) was established in 1983 as a section of the Laboratory of Human Genetics - Galliera Hospital in Genoa. Since then, samples from subjects affected by genetic disorders (and their relatives), or affected by rare diseases, have been collected and stored. The biobank takes advantage of the activity of the laboratory, skilled in pre-natal and post-natal diagnosis of chromosomal and genic disorders and exploits the lab technologies and staff experience to collect samples well characterized by cytogenetic and/or molecular analyses. Since 2008, the biobank is part of the Telethon Network of Biobanks and collaborating with the research infrastructures BBMRI Biobanking and Biomolecular Resources Research Infrastructure (www.bbmri.eu) and in 2011 it has become a new partner of EUROBIOBANK (European Network of DNA, Cell and Tissue banks for Rare Diseases - http://www.eurobiobank.org/).
The expertise of the biobank was the staring point to the new activity of Generating disease-specific induced pluripotent Stem Cells (iPSCs) for genetic disease modelling.
The example reported is related the Neutral Lipid Storage Disease with Myopathy (NLSD-M) that is a rare autosomal recessive disorder characterized by an abnormal intracellular accumulation of triacylglycerol into cytoplasmic lipid droplets (LDs). In most tissues the lipid droplets (LDs) are cellular organelles for the triacylglycerol storage. LDs metabolic functions are mediated by proteins bound to their surface. In particular, the lipase that catalyzes the removal of the first acyl chain from triacylglycerol is the patatin-like phospholipase domain-containing protein 2 (PNPLA2). This protein is coded by the PNPLA2 gene. PNPLA2 mutations cause the onset of Neutral Lipid Storage Disease with Myopathy. NLSD-M patients are affected by progressive myopathy, cardiomyopathy and hepatomegaly. Other clinical symptoms may include diabetes, chronic pancreatitis and short stature. NLSD-M has, at present, no specific therapy. We have previously reported clinical and genetic findings of some NLSD-M patients and have obtained dermal biopsies from them. Here we report the development of hiPSc (human induced pluripotent stem cell) from patients' fibroblasts harboring different PNPLA2 mutations. Initial hiPSc colony selection was based on morphologic evaluation and on detection of pluripotency surface markers (SSEA-4 and TRA-1-81). HiPSc also expressed undifferentiated ES cell markers (NANOG, SOX2 and OCT4). Karyotypic analysis of hiPSc lines indicated a normal complement of chromosomes. Immunohystochemical evaluations of LDs on hiPSc revealed that they recapitulate pathological hallmark of the disease. We propose use of differentiated cells derived from hiPSc to study the pathogenetic mechanisms leading to NLSD-M and as a cellular model for therapeutic evaluation.

## Induced pluripotent stem cell for studying development and diseases

Elvassore Nicola

Università degli Studi di Padova, Italy

The development of human organs-on-chips, in which the microscale engineering technologies are combined with cultured human cells to recapitulate the whole living organ microenvironment, offers a unique opportunity to study human physiology and pathophysiology in an organ-specific context.
This technological perspective could provide an effective solution to the limitations of conventional cell culture models that fail to recapitulate complex, organ-level disease processes in humans and it could overcome the use of costly and time-consuming animal testing, which often shows poor predictive power of human biology and physiology.
We explored whether we can control somatic human cell reprograming, stem cell expansion, selective germ layer commitment and derivation of functional tissue-specific cells on a chip through a multi-stage microfluidic-based technology. The different developmental stages required cell niche specification in terms of accurate balance between extrinsic and intrinsic cell signaling.
Human cardiomyocytes and hepatocytes generated on chip from human pluripotent stem cells show functional differentiation and proper response to drug pharmaco-kinetics, which opens a new perspective for multi-parametric and large scale human organ-based screening assays.

# Mesenchymal Stem Cells in amyotrophic lateral sclerosis

Fagioli Franca[1], Mareschi, Katia[1,2], Rustichelli Deborah[1], Ferrero Ivana[1,2], Mazzini Letizia[3], Vercelli Alessandro[4]

[1]City of Science and Health of Turin, Regina Margherita Children's Hospital, Paediatric Onco-Hematology, Stem Cell Transplantation and Cellular Therapy Division, Turin, Italy
[2]Department of Public Health and Pediatrics, University of Turin, Italy
[3]Neuroscience Institute Cavalieri Ottolenghi, Department of Neuroscience, University of Torino, Turin, Italy
[4]ALS Centre Department of Neurology, University of Eastern Piedmont, Novara, Italy

Currently, there is no treatment for Amyotrophic lateral sclerosis (ALS), a devastating neurodegenerative disease that targets motor neurons causing premature death.
Mesenchymal stem cells (MSCs) might be suitable for cell therapy in ALS because of their immunomodulatory and protective properties.
The administration of bone marrow (BM)-derived MSCs has led to beneficial effects in animal models for several neurodegenerative diseases, such as in SOD1-G93A mice, where expanded MSCs can survive and migrate after transplantation and prevent astrogliosis and microglial activation.
We performed two Phase I trials (in 2001 and 2003) for the assessment of the feasibility and toxicity of transplantation of autologous MSCs into the spinal cord in ALS patients. The trials, approved and monitored by the National Institute of Health and by the Ethics Committees, enrolled 19 ALS patients (11 M and 8 F). Patients were followed up for 6-9 months and then treated with autologous BM-MSCs. In addition to the clinical measures, we also analyzed behavioral and quality of life changes.
Eight patients died after a mean survival time of 31.6 (+21 SD) months from surgery (Range: 9-74). All deaths were deemed to be unassociated with the experimental treatment. The most important result of our studies is the demonstration of the lack of tumor formations or abnormal cell growth. All patients well tolerated the procedure, the side effects related to the surgery were mild and transient, and there was no deterioration in psychosocial status of patients. Our results showed the safety of MSC transplantation in the central nervous system during a follow-up of nearly 12 years, supporting the MSC-based therapy for neurodegenerative disorders.
More recent studies showed that the administration of human MSCs in the cisterna lumbaris of SOD1 G93A mice, providing evidence that this injection can exert strongly positive effects and could be used in outpatient surgery.

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# Bone marrow-derived mesenchymal Stem Cells reduce arteriotomy-induced stenosis in rat carotids

Finicelli Mauro[1*], Rinaldi Barbara[2,3*], Donniacuo Maria[2], Di Bernardo Giovanni[4], Del Gaudio Stefania[4], Forte Amalia[4], Peluso Gianfranco[1], Cipollaro Marilena[4], Rossi Francesco[2,3], Galderisi Umberto [4]

[1]Institute of Biomedicine and Bioresources (IBBR), CNR, Naples, Italy
[2]Department of Experimental Medicine, Section of Pharmacology "L. Donatelli", Centre of Excellence for Cardiovascular Diseases, Second University of Naples, Italy
[3]Department of Experimental Medicine, Section of Pharmacology L. Donatelli, Regional Centre for Pharmacovigilance and Pharmacoepidemiology, Second University of Naples, Italy
[4]Department of Experimental Medicine, Biotechnology and Molecular Biology Section, Second University of Naples, Naples, Italy

Restenosis following vascular injury remains a pressing clinical problem. Mesenchymal stem cells (MSCs) promise as a main actor of cell-based therapeutic strategies. The possible therapeutic role of MSCs in vascular stenosis in vivo has been poorly investigated so far. We tested the effectiveness of allogenic bone marrow-derived MSCs in reduction of stenosis in a model of rat carotid arteriotomy. MSCs were expanded in vitro retaining their proliferative and differentiation potentiality. MSCs were able to differentiate into adipocyte and osteocyte mesenchymal lineage cells, retained specific antigens CD73, CD90, and CD105, expressed smooth muscle alpha-actin, were mainly in proliferative phase of cell cycle and showed limited senescence. WKY rats were submitted to carotid arteriotomy and to venous administration with 5X106 MSCs. MSCs in vivo homed in injured carotids since 3 days after arteriotomy but not in contralateral uninjured carotids. Lumen area in MSC-treated carotids was 36% greater than in control arteries and inward remodeling was limited in MSC-treated carotids.) 30 days after arteriotomy. MSC treatment affected the expression level of inflammation-related genes, inducing a decrease of IL-1b and Mcp-1 and an increase of TGF-b in injured carotids at 3 and 7 days after arteriotomy. Taken together, these results indicate that allogenic MSC administration limits stenosis in injured rat carotids and plays a local immunomodulatory action.
In a subsequent study, we evalauted a potentially effective non-invasive strategy to mimic the MSC-mediated recovery of injured vessels. To this end we decided to assess the effectiveness of granulocyte-colony stimulating factor (G-CSF) in mobilizing endogenous mesenchymal stem cells (MSCs) and vascular progenitor cells. WKY male rats were submitted to carotid arteriotomy and submitted to a nine days treatment (3 days pre- to 6 days post-arteriotomy) with G-CSF (19 mug/Kg/die) or saline. Carotids were harvested 7 and 30 days following arteriotomy (early- and late-phase, respectively) and MSCs were isolated from the bone marrow of the same animals; histological, molecular and cellular analysis were conducted to dissect the effect of G-CSF treatment in vascular (re)stenosis. Statistical significance was determined using two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Although morphometric analysis did not reveal differences in lumen narrowing between G-CSF- and CTRL-carotids 30 days following arteriotomy, we detected a noticeable conservative effect of G-CSF treatment on vascular wall morphology. Histological and molecular analyses disclosed an increase in cellularity within the tunica media with a concomitant increase of the VSMCs differentiation markers both at early- and late-phases of (re)stenotic response in G-CSF-treated carotids (Sm22-alpha, Myocd and Smtn). These findings were accompanied by data about the downregulation of oxidative stress-related genes (Hif-1alpha, Vegf and Vegfr2) in G-CSF-injured rats along with an increased clone forming potential of MSCs harvested from the same group. The effect exerted by G-CSF in our model of arteriotomy-induced (re)stenosis seemed to consist in supporting the recovery of the architecture of the tunica media of injured vessels by: i) inducing VSMCs differentiation; ii) limiting the oxidative-stress response induced by arteriotomy; iii) promoting the healing activity of MSC secretome. In conclusion, G-CSF treatment may only partially mimic the therapeutic effect of MSC transplant in stenosis disease.

## Mesenchymal Stem Cells: paracrine mechanisms in heart repair

Gnecchi Massimiliano

University of Pavia, Department of Molecular Medicine, Unit of Cardiology, Pavia, Italy

Acute myocardial infarction (AMI) represents a significant cause of mortality and morbidity worldwide. Following AMI, cardiomyocytes begin to die, and if the blood supply is not quickly restored, all of the cardiac tissue served by the related infarcted artery undergoes necrosis or apoptosis, leading to chronic sequelae of ischemic cardiomyopathy and congestive heart failure. The endogenous regenerative capacity of the heart seems unable to replenish a significant loss of tissue such as that resulting from AMI. However, the recent discovery of resident cardiac stem cells (CSCs), together with the demonstration of bone marrow (BM)-derived stem cells able to home in the heart and transdifferentiate into cardiomyocytes, has suggested the fascinating possibility that therapeutic myocardial regeneration might be achieved using adult stem cells (ASCs). To date, the majority of animal and preliminary human studies of ASC therapy following AMI have demonstrated an overall improvement in cardiac function.

Myocardial and vascular regeneration were initially proposed as mechanisms of stem cell action. However, the frequency of stem cell engraftment and the number of newly generated cardiomyocytes and vascular cells appear too low to explain the significant cardiac improvement observed. Instead, there is a growing body of evidence supporting the hypothesis that paracrine mechanisms mediated by factors released by the ASCs play an essential role in the reparative process observed after stem cell injection into infarcted hearts. It has been shown that ASCs, particularly mesenchymal stromal cells (MSCs), produce and secrete a broad variety of cytokines, chemokines, and growth factors that are involved in cardiac repair. The paracrine factors influence adjacent cells and exert their actions via several mechanisms. Myocardial protection and neovascularization are the most extensively studied. Furthermore, the postinfarction inflammatory and fibrogenic processes, cardiac metabolism, cardiac contractility, and/or endogenous cardiac regeneration may also be positively influenced in a paracrine fashion.

# MSCs in bone and cartilage repair

Jones Elena

Leeds Institute of Rheumatic and Musculoskeletal Medicine, UK

Cell therapy with MSCs has been increasingly used for the treatment of bone and cartilage defects. Most commonly, MSCs are manufactured by culture-expansion of plastic adherent cells from bone marrow (BM) aspirates or from lipoaspirates. For large bone defect reconstruction MSCs are normally seeded on osteoconductive scaffolds, which can be either natural or synthetic. In cartilage repair applications, MSCs are normally delivered intra-articularly or injected under a barrier membrane to prevent their loss in the joint. Alternative strategies rely on manipulating resident MSC homing towards the defect area with the use of smart scaffolds loaded with chemotactic molecules. Although these latter strategies, which are based on minimal manipulation of autologous MSCs, are quite attractive from a regulatory point of view, there remain many unresolved issues.

In terms of repairing bone in healthy individuals after an acute trauma, the main issue appears to be the quantity rather than quality of autologous MSCs. In clinical settings, autologous BM MSCs are concentrated by centrifugation, but their purity and dose remain unknown at the time of surgery. In our laboratory, we have developed methods for prospective quantification of MSCs in BM aspirates and concentrates based on the CD271 marker. We have also shown that larger numbers of autologous MSCs could be recovered in reaming waste bags and can be selected, to a high degree of purity, using a GMP-compatible CliniMacs- based technology. Human MSCs cannot be mobilized into peripheral blood; however our recent work analyzing a large cohort of patients at two time-points post-fracture shows that their BM MSC levels fluctuate in direct correlation with the levels of circulating platelets and their products, PDGFs. This opens up a new way of boosting BM MSC numbers, prior to their harvest, using autologous platelet-derived products. The choice of scaffold to house MSCs is also important and the addition of collagen appears to enhance MSC attachment and proliferation inside the scaffold.

In terms of repairing cartilage, the 'quality' of autologous MSCs, particularly in patients with osteoarthiritis (OA), remains a primary concern. Previous studies have indicated some abnormalities in both BM-derived and synovium-derived MSCs from OA patients and our recent work shows that MSCs in their subchondral bone, in areas directly beneath the cartilage damage, display transcriptional and functional alterations compared to MSCs from non-involved bone. These results suggest that allogeneic MSCs from healthy individuals, or autologous MSCs from non-involved sites such as lipoaspirates, could be a better choice of MSCs for repairing joint integrity in these patients. Conversely, pharmacological targeting of sunchondral bone MSCs in early OA could represent a novel way to slow the progression of this debilitating disease.

## Mesenchymal Stromal Cells: from biology to standardisation

Krampera Mauro

Stem Cell Research Laboratory, Section of Hematology, Department of Medicine, University of Verona, Italy

Mesenchymal stromal cells (MSCs) are adult non-hematopoietic cells provided of stem cell potential originally isolated from bone marrow (BM), but virtually present in all body tissues, thus giving rise to a mesenchymal-stromal cell system throughout the body. Different Authors showed that MSCs are a subgroup of vessel-lining pericytes that may contribute to vessel homeostasis by reacting to tissue damage with regenerative processes, locally modulating the inflammatory reaction. MSC capability of differentiating into a number of tissues, mainly of mesodermal origin, as well as modulating a broad number of immune effector cells belonging to both to the innate and adaptive immunity, paved the way to MSC use for clinical purposes. A number of MSC-based clinical trials were started and completed, with some reported successes in multiple clinical indications, and a growing number of companies were established. Nevertheless, many important issues still exist, including MSC definition and comparability among different MSC biological features, expansion procedures, quality controls, and clinical outcome. Thus, the need of general standardization of MSC field has become more and more urgent. International Society for Cellular Therapy (ISCT), together with other international societies worldwide, is trying to go further MSC definition criteria established in 2005 and 2006 to harmonize the general approach to MSC reseach and clinical application.

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## Mesenchymal Stem Cells and Parkinsonism

Giordano Rosaria, Montemurro Tiziana, Viganò Mariele, Parazzi Valentina, Lavazza Cristiana, Budelli Silvia, Barilani Mario, Ragni Enrico, Marino Luigi, Lazzari Lorenza, Canesi Margherita, Pezzoli Gianni

Fondazione IRCCS Ca' Granda Ospedale Maggiore, Policlinico di Milano, Italy

Mesenchymal stem cells (MSCs) are multipotent cells that can be isolated from many sources. Recently, several cellular therapy products have been developed for therapeutic applications in neurodegenerative disorders in compliance with Good Manufacturing Practices (GMP). Since BM MSC production from elderly and diseased patients in the autologous context is extremely challenging, some technical aspects of cell culture should be considered (in particular culture duration and the use of FBS or other substitutes) factors. Here we describe the results of GMP procedures to obtain BMMSCs suitable for autologous use in patients affected by a rare form of parkinsonism (Progressive Supranuclear Palsy) within a specific clinical protocol (NCT01824121). A potency assay, based on the secretion of the two major neurotrophic factors (BDNF and GDNF) putative mediators of MSC neuroprotection have been developed. The target cell dose of 1.5 + 0.5 x 10E6 cells was reached in 5/9 patients. The quality controls (MSC-specific immunophenotype, viability →80%, absence of bacterial, fungi and mycoplasma contamination, low endotoxin level, normal karyotype) were conform in all the preparations. This study supports the concept that each clinical trial should be designed in order to choose the production protocol that is able to obtain the best results in view of the specific clinical needs.

# S-adenosylmethionine and tyrosol protect HepG2 cells from alcohol-induced oxidative damage

Bagarolo Maria Libera, Ilisso Paola, Vanacore Daniela, Caraglia Michele, Porcelli Marina, Cacciapuoti Giovanna, Stiuso Paola

Second University of Naples
Department of Biochemistry, Biophisics and General Pathology
Naples, Italy

A consequence of chronic alcohol consumption is the disregulation of methionine metabolism that causes a significant reduction in the synthesis of S-adenosyl-methionine (AdoMet), crucial methyl donor for DNA and histone methylation and a key metabolite that regulates hepatocyte growth, differentiation and death. Ethanol has been reported to inhibit the activity of a vital cell enzyme, methionine synthase thus leading to a decrease of AdoMet, and to an increase of S-adenosylhomocysteine, potent competitive inhibitor of transmethylation reactions and homocysteine (Hcy) formation. In the liver, AdoMet plays also an important role in regulating the levels of glutathione, the major endogenous antioxidant involved in the pathogenesis of alcoholic liver disease. Finally, a redox-sensitive class III histone deacetylase molecule, SIRT1 is also decreased in rat hepatocytes exposed to alcohol and in livers of alcohol-fed rats. Several studies reported that many plant-derived phytochemicals compounds exert their protective effects through mechanisms not strictly related to their scavenging properties. On this light, data from our laboratory suggested that tyrosol (Tyr) down-regulates ICAM-1 expression induced by Hcy in EA.hy 926 cells. In the present study, we investigated the hepatoprotective activity of AdoMet and Tyr combinations against ethanol-induced oxidative stress in human hepatoma HepG2 cells and we explored the potential molecular mechanisms through which the protective effect of these molecules is exerted. Higher ethanol concentrations (1 M for 4 h) were used to induce oxidative damage in HepG-2 cells. The cells were then treated with Ado-Met/Tyr combination and the cellular damage was assessed. The combination inhibited the overproduction of malondialdehyde and reactive oxygen species, normalized ferritin and Hcy levels and increased Sirt1 expression. In conclusion, our findings indicate that the hepatoprotective actions of Ado-Met/Tyr combination may be mediated through the modulation of oxidative stress and involves SIRT1 up-regulation.

# Prognostic role of LGALS3BP in Ewing Sarcoma

Baricordi Cristina, Grilli Andrea, Picci Piero, Scotlandi Katia

Orthopaedic Rizzoli Institute
Laboratory of Experimental Oncology, CRS Development of Biomolecular Therapies
Bologna, Italy

*Objective*
LGALS3BP, a secretory glycoprotein implicated in cell-cell and cell-extracellular matrix interaction, has been associated with progression and metastasis in a variety of human cancers. By means of adequate cellular models we previously described a possible oncosuppressor role for LGALS3BP in Ewing Sarcoma (EWS), which expression affected cell adhesion and mobility in vitro, as well as metastasis in vivo. We also uncovered its value as a biological indicator of prognosis in a cohort of 56 patients. Based on these evidences, the primary objective of this study was to validate LGALS3BP prognostic relevance in a larger group of EWS patients.

*Materials and Methods*
Quantitative RT-PCR was preformed using TaqMan® Gene Expression Assays (Applied Biosystems) on 109 primary tumors from non-metastatic EWS patients treated at the Rizzoli Institute with neoadjuvant chemotherapy. Normal mesenchymal stem cells and pooled normal muscle were used as calibrators. Log rank test, Kaplan-Meier survival curves and Cox multivariate analyses were used as statistical methods.

*Results*
High expression of LGALS3BP in localized tumors was significantly related to better event-free (EFS) and overall survival (OVS) (p= 0.002 and 0.0290, respectively). By multivariate Cox regression analysis, high LGALS3BP expression was confirmed as an independent risk factor associated with a more favourable outcome (EFS: RR= 0.336 - 95%CI= 0.154-0.732 ; OVS: RR= 0.352 - 95%CI= 0.147-0.847). Prospective evaluation of LGALS3BP as a prognostic biomarker is ongoing.

*Conclusions*
LGALS3BP expression level confirmed to be a predictor of clinical outcome in EWS. We propose that routine evaluation of LGALS3BP could help stratify patients according to different risk at diagnosis, possibly sparing excessive long-term toxicity to those with markers of good prognosis.

# Characterization of cell damage and death response in human cybrid models of Parkinson disease

Basello Davide[1], Zanetta Francesca[1], Pignataro Daniela[1], Bottone Maria Grazia[2], Prosperi Ennio[1], Scovassi A. Ivana[1], Montecucco Alessandra[1], Biamonti Giuseppe[1]

[1]Istituto di Genetica Molecolare CNR, Pavia, Italy
[2]Dipartimento di Biologia e Biotecnologie "L. Spallanzani", Università di Pavia, Italy

*Objective*
Consistent findings indicate that mitochondrial dysfunction and the consequent accumulation of ROS/oxidative damage are closely linked to Parkinson Disease (PD). Several mitochondrial features appear to be affected in this disease, including biogenesis, bioenergetics, dynamics, transport and quality control. The aim of our work is to study oxidative damage/stress response and signal transduction pathways in a PD cellular context.

*Materials and Methods*
Material: Human cytoplasmic hybrid cell lines (cybrids) (kindly supplied by Dr. Patricia Trimmer, Virginia Commonwealth University, Richmond, USA) obtained by fusing platelets containing mitochondria from PD patients with host mtDNA-free SH-SY5Y neuroblastoma cells. Three PD cybrids and three control cybrids were used.
Methods: Cybrids were treated with increasing concentrations (10-400 μM) of terbutylhydroperoxide (TBOOH) to measure their survival by MTT assay, microscopy and flow cytometry. Typical markers of apoptosis (PARP-1 proteolysis) or autophagy (presence of LC3 form II) were investigated by western blot. Mitochondria distribution and functionality were monitored by mtHSP70 localization and JC-1 staining, respectively.

*Results*
Survival experiments indicated that PD cybrids were more sensitive to TBOOH compared to the control ones. Preliminary studies revealed that cell death occurred without the typical features of apoptosis or autophagy. The activation of a neuron-specific cell death (called parthanatos), involving the extrusion of crucial factors from depolarized mitochondria, was suggested by JC-1 staining.

*Conclusion*
Our results support an active role of mitochondria impairment in PD cybrids, which we found to be more susceptible to oxidative stress and more affected in mitochondrial dynamics than control cybrids. Of note, a decrease in cell survival was paralleled by the activation of a peculiar form of death, named parthanatos, which involves essentially the extrusion of crucial factors from the depolarized mitochondria.
This work is in the frame of the Accordo Quadro Regione Lombardia/CNR (2013-2015) Metodologie di base per l'innovazione della diagnosi e nella terapia di malattie multifattoriali (MbMM). DB and FZ are funded by fellowships from MbMM.

# Evaluation of a novel electroporation system in cisplatin chemotherapy: in vitro and in vivo studies

Bitti Giuseppe[1], Baldi Alfonso[2], Boccellino Mariarosaria[1], Quagliuolo Lucio[1]

[1]Seconda Università degli Studi di Napoli
Dipartimento di Biochimica, Biofisica e Patologia Generale, Napoli, Italy
[2]Seconda Università degli Studi di Napoli
Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche, Caserta, Italy

*Objective*
The purpose of this work was to evaluate the efficacy of a new electroporator (Onkodisruptor) in cisplatin-based chemotherapy, which differs for some features of the pulse compared to those used by electroporators most common currently used in chemotherapy. In particular, this new system is characterized by a smaller interval between the electric pulses and a lower total treatment time. The studies were carried out both in vitro, in a cellular model, A549 (human lung carcinoma), that in vivo by implanting the tumor in BALB/c nude mice.

*Materials and Methods*
In vitro, the cell proliferation of A549 cells was evaluated by MTT assay while the apoptosis was assessed using an assay based on a double staining Annexin V-FITC and propidium iodide (PI) and results were analyzed using flow cytometry. In vivo experiments were conducted on 20 mice that were administered with a solution of cancer cells. The mice were divided into 2 groups: a first group treated with cisplatin after standard electroporation treatment; a second group treated with cisplatin after experimental electroporation with Onkodisruptor.

*Results*
The in vitro experimental results have shown a significant increase of apoptosis when the cells were first electroporated with the new system and then treated with cisplatin. The results obtained in vivo have shown both the inhibition of tumor growth, that the almost complete destruction of the neoplastic tissue confirmed by histological examination, in the samples in which the electroporation was performed with the Onkodisruptor. Finally, animals treated with protocol had a lower degree of muscle contraction compared to those treated with standard protocol.

*Conclusion*
In conclusion, this work can be a basis for the design of a new scheme of electrochemotherapy, which enhances the advantages, prolonging survival, improving the quality of life and minimizing side effects.

# In vitro inhibition of U87 mg glioblastoma cell growth by CD14+ cells primed with paclitaxel

Bonomi Arianna[1], Lisini Daniela[2], Navone Stefania Elena[3], Frigerio Simona[2], Dossena Marta[4], Ciusani Emilio[5], Rampini Paolo[3], Marfia Giovanni[3], Coccè Valentina[1], Cavicchini Loredana[1], Sisto Francesca[1], Parati Eugenio[4], Mantegazza Renato[6], Rimoldi Marco[7], Rizzetto Manuela[7], Alessandri Giulio[4], Pessina Augusto[1]

[1]Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy
[2]Cell Therapy Production Unit, Fondazione IRCCS Neurological Institute C. Besta, Milan, Italy
[3]Laboratory of Experimental Neurosurgery and Cell Therapy, Neurosurgery Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy
[4]Cellular Neurobiology Laboratory, Department of Cerebrovascular Diseases; Fondazione IRCCS Neurological Institute C. Besta, Milan, Italy
[5]Laboratory of Clinical Pathology and Neurogenetic Medicine, Fondazione IRCCS Neurological Institute C. Besta, Milan, Italy
[6]Unit of Neuro-Immunology, Fondazione IRCCS Neurological Institute C. Besta, Milan, Italy
[7]Division of Biochemistry and Genetics, Fondazione IRCCS Neurological Institute C. Besta, Milan, Italy

*Objective*

In the attempt to develop new strategies to circumvent the immunosuppression associated with glioblastoma (GB), novel approaches have been designed by using dendritic cells (DCs)-based vaccination, considered a promising strategy to attack high-grade glioma. Besides the use of DCs for GB immunotherapy, peripheral blood (PB)-derived CD14+ and DCs have also been engineered for deliver anti-cancer molecules because, such as Mesenchymal Stromal Cells (MSCs), home tumour microenvironment. We've have previously demonstrated that human MSCs without any genetic manipulation but only primed with Paclitaxel (PTX), acquired anti-tumour activity, providing a new biological approach for drug delivery. Based on these results, we investigated whether both CD14+ and their derived DCs may behave like MSCs acquiring anti-tumour activity upon PTX priming.

*Materials and Methods*

Human CD14+ cells, isolated from PB by immunomagnetic selection, were differentiated into mature DCs. CD14+ and DCs purity was checked by cytofluorimetric analysis. CD14+ and DCs were primed by incubation with 1 µg/ml of PTX for 24h. The effects of the conditioned media (CM) from PTX primed CD14+cells (CD14+PTX-CM) and Dendritic Cells (DCsPTX-CM) were evaluated in a standard assay on leukemia cells. The release of PTX into the CM was confirmed by mass spectrometry. Direct anti-tumour activity was investigated by a co-culture assay with CD14+PTX cells and human glioblastoma cell line U87 MG.

*Results*

Both CD14+ and DCs incorporate PTX and release the drug in the CM in time-dependent manner. The addition of CM from CD14+ or DCs loaded with PTX strongly inhibit the proliferation of leukemia cells. The co-colture of CD14+PTX with U87 MG show the ability of CD14+PTX to directly inhibit in vitro tumor cell proliferation.

*Conclusion*

Our results are the first demonstration that, in GB therapy, PB-derived CD14+ and DCs could be used not only for immunotherapy but also to delivery anti-cancer drugs, such as PTX, into the tumor site.

# Effect of Tributyltin on porcine endothelial cell integrity

Botelho Giuliana[1], Bernardini Chiara[2], Zannoni Augusta[2], Bianchi Francesca[3], Ventrella Vittoria[2], Forni Monica[2]

[1]UNICENTRO – Universidade Estadual do Centro-Oeste do Paraná, Brazil
[2]University of Bologna, Department of Veterinary Medical Sciences – DIMEVET Bologna, Italy
[3]Laboratory of Molecular Biology and Stem Cell Engineering - National Institute of Biostructures and Biosystems, Department of Experimental, Diagnostic and Specialty Medicine, S. Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy

*Objective*

Many physiological imbalances are nowadays attributed to toxic compounds widespread in the environment, among these, organotins, including tributyltin (TBT), exert generic cytotoxic effect including oxidative stress but also tissue-specific effects such as endocrine disruptors. TBT was already found in human blood and accumulated in endothelial cells at mitochondrial level. A correlation between TBT and cardiovascular disease it has been hypothesized but incompletely demonstrated. To date very few studies analyzed the specific effects of TBT on endothelial cells, therefore in the present research we evaluated the effect of TBT on porcine aortic endothelial cells (pAECs).

*Materials and Methods*

pAECs were incubated with increasing dose of TBT (0, 100, 250, 500, 750, 1000 nM) to evaluate cytotoxicity and apoptosis, and with TBT (100, 500 nM) for 1, 7, 15h to study the expression of some genes involved in endothelial integrity and in the inflammatory response.

*Results*

TBT reduced cell viability in a dose dependent manner , the flow cytometric data showed that TBT induced both apoptosis and necrosis with early apoptosis prevalent at the lowest doses and earliest times. Adhesion molecules exhibited a different response to TBT exposure, VCAM-1 was significantly reduced with both TBT concentrations starting from 7 h of exposure, ICAM-1 showed a transient decrease at early times and low dose while P-SEL significantly increased after 15 h of exposure at TBT 500 nM. TBT reduced the expression of molecules involved in cell-to-cell junctions: OCC-1 and ZO-1. IL-6 mRNA was transiently reduced at early times for both TBT doses, while after 15h of exposure at TBT 500 nM the IL-6 expression was significantly increased.

*Conclusions*

Overall we observed that TBT was toxic to pAECs and showed the ability to deeply interfere with expression of key genes involved in in the control of endothelial integrity.

## Leptin influences metabolic compliance of prostate cancer cells

Calgani Alessia, Delle Monache Simona, Angelucci Adriano

Dipartimento di Scienze Cliniche Applicate e Biotecnologiche, University of L'Aquila, Italy

Leptin is actively investigated for its direct effect on cancer cells, but little is known about the molecular mechanisms underlying its tumorigenic properties.
We investigated leptin signaling in prostate transformed epithelial cells, non-tumorigenic RWPE-1 and tumorigenic WPE1-NB26, and in carcinoma cell lines, PC3 and LNCaP.
Leptin treatment reduced ROS production and stabilized mitochondrial potential. In addition recombinant leptin stimulated production and release of lactate and sustained cell survival in presence of the inhibitor of phosphorylative oxidation, oligomycin. The lactate export is allowed by upregulation of monocarboxylate transporters MCT1 and MCT4. Moreover CD147/basigin expression, stimulated in presence of leptin, played a key role in modulation of MCTs. In fact silencing of CD147/basigin determined a reduction in both MCT1 and MCT4. In addition the inhibition of CD147/basigin reduced markedly the ability of leptin to support cell growth, both in standard condition and in presence of olygomicin.
Leptin action, through the modulation of cancer metabolism, may explain, at least partially, the association between obesity and prostate cancer. The proposed mechanism, involving new targetable intermediates, such as MCTs and CD147/basigin, offers new opportunities in diagnosis stratification and treatment for prostate carcinoma patients with high serum leptin levels.

# Role of Src inhibition in modulating ROS production in prostate cancer

Calgani Alessia[1], Schenone Silvia[2], Angelucci Adriano[1], Botta Maurizio[3], Delle Monache Simona[1]

[1]Dipartimento di Scienze Cliniche Applicate e Biotecnologiche, University of L'Aquila, Italy
[2]Dipartimento di Farmacia, University of Genoa, Italy
[3]Dipartimento di Biotecnologie, Chimica e Farmacia, University of Siena, Italy

Prostate cancer cells frequently express over-activated protein kinase Src. Although reactive oxygen species (ROS) are key mediators of Src tumorigenic properties, few is known about their role in prostate cancer resistance to therapy.
We evaluated, in vitro and in vivo, in a hormone-insensible prostate cancer (PCa) cell model the effect of S13, a tyrosine kinase inhibitor with a prevalent specificity for Src in combination with paclitaxel (PTX), a mitotic inhibitor used in cancer chemotherapy.
In vivo combination treatment with PTX and S13 reduced dramatically PCa tumor growth with a relevant difference in the density of new blood vessels respect to control and single treatments. This reduction was determined by a concomitant impairment of endothelial cells migration and of VEGF release by cancer cells. In fact S13, when used alone, was sufficient to reduce tubule formation in vivo, and to inhibit VEGFR2 activation and FAK expression in endothelial cells. The combination treatment determined a significant reduction in ROS production and HIF1 stabilization in PCa cells respect to single treatments with S13 or PTX.
Src-inhibition could be an effective adjuvant therapy aimed to control pro-tumoral production of ROS in aggressive PCa.

# Heat shock protein 60 (Hsp60) modulation by the Histone Deacetylase Inhibitor (HDAC-i) SAHA in mucoepidermod tumor H292 cells

Campanella Claudia[1,2], Caruso Bavisotto Celeste[1,2], Marino Gammazza Antonella[1,2], Lo Cascio Filippa[1,2], Emanuele Sonia[1], Danneo Antonella[3], Lauricella Marianna[1], Cappello Francesco[1]

[1]Department of Experimental Biomedicine and Clinical Neurosciences, University of Palermo, Italy
[2]Euro-Mediterranean Institute of Science and Technology, Palermo, Italy
[3]Department of Biological, Chemical and Pharmaceutical Sciences and Technologies, University of Palermo, Italy

*Objective*
Hsp60 is a mitochondrial chaperone essential for mitochondrial protein folding. Hsp60 over-expression has an important role in cancer development and progression. HDACi are epigenetic drugs that modulating gene expression induce cell death in different tumor models. Treatment with HDACi can result in hyperacetylation of chaperones including Hsp90 and Hsp70, but no data are available about their effects on Hsp60. In this study, we investigate the effects of the HDACi SAHA on Hsp60 in H292 tumor cell line.

*Materials and Methods*
H292 cells were treated with different doses of SAHA for 24h. MTT test and flow cytometry cell cycle analysis were conducted. Mitochondrial membrane potential was evidenced using a JC-1 assay. Hsp60 expression and acetylation state were evaluated using western blot and immunoprecipitation. Hsp60 localization was analyzed by immunofluorescence. Cells were treated with SAHA and the proteasome inhibitor MG132 to elucidate whether Hsp60 undergoes proteasomal degradation under the SAHA effect.

*Results*
After SAHA treatment dose-dependent reduction of cell viability was observed, after 24h the cells were arrested in the G2/M phase, whereas pre G0/G1 peak appeared at 48 h indicating DNA fragmentation. Moreover SAHA determined a loss of mitochondrial membrane potential and preliminary results seem to indicate that this event is most likely correlated with new Hsp60 localization. Treatment with SAHA reduced significantly Hsp60 levels and seemed to favour its acetylation. Instead, Hsp60 levels did not change after combined treatment with SAHA and MG123.

*Conclusion*
SAHA induces growth suppression of H292 cells and decreases the level of Hsp60. This reduction could be due in part to Hsp60 proteolitic degradation in part to its externalization from the mitochondia and possible extracellular export. This hypothesis will be further investigated. SAHA seems to interfere with the pro-tumoral role of Hsp60 which could be then considered as a novel target in cancer therapy.

# Chemosensitization of breast cancer cells to chemotherapy through HIF-1alpha inhibition in hypoxic environment: preliminary studies

Chiavegato Giulia [1], Sega M.[2], Carcereri de Prati A.[1], Butturini E.[1], Chignola R.[2], Mariotto S.[1]

[1]Department of Life and Reproduction Sciences, Biochemistry Section, University of Verona, Italy
[2]Department of Biotechnology, University of Verona, Italy

*Objective*

The hypoxic environment of solid tumors induces adaptive changes of tumor cells that are associated with a more metastatic phenotype and a higher chemoresistance to conventional treatment. The transcription factor HIF-1 is a master regulator of the response of cells to low oxygen levels and it mediates the expression of many genes involved in the regulation of glycolysis, glucose transport, cell survival and proliferation, chemoresistance, angiogenesis and metastasis. HIF-1 activity is deregulated in many human cancers due to the overexpression of HIF-1alpha, the regulatory subunit of HIF complex, or to the constitutive activation of STAT3, a transcription factor upstream of HIF-1.
Recently, we have identified pro-apoptotic plant extracts and natural compounds as potent anti-STAT3 agents.
On the basis of these results, the ability of these compounds to induce cell death and to sensitize tumor cells to standard chemotherapeutic agents under hypoxic conditions, were analyzed.

*Materials and Methods*

Two in vitro experimental cell models of hypoxia were used: breast cancer MDA-MB231 cell line cultured in hypoxic chamber and breast cancer T47D cell line grown as multicellular spheroids. Cells proliferation was determined by WST or ATP assays. HIF-1 and STAT3 expression were evaluated by Western Blot.

*Results*

Cell cultured under hypoxia and as spheroids are characterized by higher STAT3 phosphorylation and HIF-1alpha expression.
Cynara scolymus or Helichrysum angustifolium extracts as well as alpha-bisabolol decreased tyr705phosphorylation of cytosolic STAT3 and HIF-1alpha expression after 24 hours treatment. Furthermore, alpha-bisabolol inhibited spheroids proliferation and decreased spheroid compactness.

*Conclusion*

Our preliminary results suggest that these experimental models allow to study in vitro some of the important features of the tumor microenvironment and that the identified plant extracts retain their anti-STAT3 activity also under hypoxic conditions, thus decreasing HIF-1 alpha expression. This approach shows up as a good starting point to test the effectiveness of combination chemotherapy.

# Modulation of osteosarcoma and melanoma cells drug-resistance by not immunotoxic natural compound

Condello Maria[1,2], Pellegrini Evelin[1], Cosentino Dario[1], Corinti Silvia[3], Di Felice Gabriella[3], Multari Giuseppina[4], Gallo Francesca Romana[4], Giuseppe Arancia[1], Meschini Stefania[1]

[1]National Institute of Health, Dept. Technology and Health, Rome, Italy
[2]National Research Council, Institute of Chemical Methodologies, Rome, Italy
[3]National Institute of Health, Dept. Infectious, Parasitic and Immune-Mediated Diseases, Rome, Italy
[4]National Institute of Health, Dept. of Therapeutic Research and Medicines Evaluation, Rome, Italy

The main goal of this research is to prove on hystologically different resistant cancer cells the modulatory effect of the plant alkaloid voacamine (VOA) on the doxorubicin (DOX) toxicity. Moreover, the non-toxicity in normal human cells and on mouse bone marrow-derived dendritic cells (BM-DC) is deepened. We used cell cultures technique, optical and transmission microscopy, MTT assay and flow cytometry .

The in vitro investigations herein reported, showed the following results: (i) the chemosensitizing effect of VOA on SAOS-2-DX cells, an osteosarcoma resistant cell line P-glycoprotein (P-gp) overexpressing, in terms of drug accumulation and cell survival evaluation;

(ii) the plant alkaloid enhanced the cytotoxic effect of DOX also on metastatic melanoma cell line (Me30966), intrinsically drug resistant and P-gp surface negative;

(iii) at the concentrations used to sensitize tumor cells, VOA was not cytotoxic on human fibroblasts ;

(iv) in vitro VOA treatment of BM-DC, neither directly affected their maturation nor influenced LPS-induced maturation, indicating the absence of intrinsic immunotoxic and inflammatory properties of the natural compound.

This natural and conventional drug combination may be a winning strategy for a high therapeutic doses reducing and for an anticancer therapy optimizing.

# Mutant p53 stimulates chemoresistance of pancreatic adenocarcinoma cells to gemcitabine

Cordani Marco[1], Fiorini Claudia[1], Padroni Chiara[1], Blandino Giovanni[2], Di Agostino Silvia[2], Donadelli Massimo[1]

[1]University of Verona, Department of Life and Reproduction Sciences, Section of Biological Chemistry, Verona, Italy
[2]Translational Oncogenomics Laboratory, Regina Elena National Cancer Institute, Rome, Italy

Pancreatic adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths worldwide characterized by poor prognosis, resistance to conventional chemotherapy and a very high mortality rate. TP53 tumor suppressor gene is frequently mutated in PDAC, resulting in the accumulation of mutated protein with potential gain-of-function (GOF) activities, such as genomic instability, hyperproliferation and chemoresistance. The purpose of this study was to assess the relevance of the p53 status on the PDAC cells response to the standard drug gemcitabine. We also examined the potential therapeutic effect of p53-reactivating molecules to restore the mutant p53 function in GEM treated PDAC cells. We showed that gemcitabine stabilized mutant p53 protein in the nuclei and induced chemoresistance, concurrent with the mutant p53-dependent expression of Cdk1 and CCNB1 genes, resulting in a hyperproliferation effect. Despite the adverse activation of mutant p53 by gemcitabine, simultaneous treatment of PDAC cells with gemcitabine and p53-reactivating molecules (CP-31398 and RITA) reduced growth rate and induced apoptosis. This synergistic effect was observed in both wild-type and mutant p53 cell lines and was absent in p53-null cells. The combination drug treatment induced p53 phosphorylation on Ser15, apoptosis and autophagosome formation. Furthermore, pharmacological inhibition of autophagy further increased apoptosis stimulated by gemcitabine/CP-31398 treatment. Together, our results show that gemcitabine aberrantly stimulates mutant p53 activity in PDAC cells identifying key processes with potential for therapeutic targeting. Our data also support an anti-tumoral strategy based on inhibition of autophagy combined with p53 activation and standard chemotherapy for both wild-type and mutant p53 expressing PDACs.

# CXCR4/CXCL12/CXCR7 and TLR2-4 mediated inflammation in Colorectal Liver Metastases

D'Alterio Crescenzo[1], Nasti Guglielmo[2], Polimeno Marianeve[1], Circelli Luisa[1], Ottaiano Alessandro[2], Tatangelo Fabiana[3], Izzo Francesco[4], Iaffaioli Rosario Vincenzo[2], Scala Stefania[1]

[1]Istituto Nazionale Tumori, IRCCS Fondazione "G. Pascale", Oncological Immunology, Naples, Italy
[2]Gastrointestinal Medical Oncology, National Cancer Institute "G. Pascale", Naples, Italy
[3]Pathology, National Cancer Institute "G. Pascale", Naples, Italy
[4]Hepatobiliary Surgery, National Cancer Institute "G. Pascale", Naples, Italy

Approximately only 25% of colorectal cancer (CRC) patients with liver-limited metastasis (CRLM) are resectable with curative intent. Use of first-line appropriate conversion-therapy may allow subsequent resection in a further 24–54% of initially unresectable patients. Emerging data suggest that chemokine CXCL12 and its cognate receptors CXCR4 and CXCR7 affects proliferation/migration and immunoescape in CRC. A key signaling of inflammation utilizes Toll-like receptor (TLR) family, recognizing endogenous macromolecules released by injured tissue. During tumorigenesis TLR-driven tissue response may promote neoangiogenesis and proliferation.

*Objective*
To evaluate the role CXCR4-CXCL12-CXCR7 and TLR 2-4 in CRLM patients. To evaluate the prognostic role CXCR4-CXCL12-CXCR7 in CRLM patients. To identify new therapeutic target for neoadjuvant and adjuvant treatment of CRLM
Materials and Methods: CXCR4-CXCL12-CXCR7, TLR2 and-4 expression was prospectively evaluated in 33 biopsies from CRLM uniformly neoadjuvant-treated according to Folfiri+Avastin-regimen (G.Nasti, Br.J.Cancer.2013) through quantitative real-time PCR, Immunohistochemistry and Western-blot.

*Results*
CXCL12 was down-expressed, while CXCR4 and CXCR7 were over-expressed in CRC-metastasis when compared to normal adjacent liver tissue; as well as TLR2 and TLR4. Interestingly CXCR4-CXCL12-CXCR7 clearly discriminated patients in risk categories. Poor survival patients profile were characterized by CXCR4 and CXCR7 over-expression with concomitant down-expression of CXCL12; high CXCL12 expression correlates with better prognosis increasing progressively in patients with absence of disease. TLR2 but not TLR4 increased in patients with worse prognosis.

*Conclusion*
CXCR4-CXCL12-CXCR7 axis evaluation has a prognostic value in CRLM suggesting CXCR4 inhibition as novel strategy in CRLM conversion therapy.

# Beneficial effect of glucose-lowering treatments on EPC number and differentiation during early percutaneous coronary intervention

D'Onofrio Nunzia[1], Marfella Raffaele[2], Vitiello Milena[1], Paolisso Giuseppe[2], Balestrieri Maria Luisa[1]

[1]Second University of Naples, Department of Biochemistry, Biophysics and General Pathology, Naples, Italy
[2]Second University of Naples, Department of Geriatrics and Metabolic Diseases, Naples, Italy

*Objective*
Injury to the heart causes endothelial progenitor cells (EPC) to migrate to the site of damage and differentiate to endothelial cells. In patients with acute myocardial infarction (AMI), EPC are rapidly recruited to myocardium where they mediate a protective effect on vascular healing and ischemic preconditioning. In diabetic patients, impaired levels and functional activities of EPC at the time of myocardial infarction occur. Here, we examined the effects of peri-procedural intensive glycemic control during early percutaneous coronary intervention (PCI) on the number and differentiation of EPC in hyperglycemic patients with first ST-elevation myocardial infarction (STEMI).

*Material and Methods*
We conducted a randomized, prospective, open label study on 194 patients with STEMI undergoing PCI: 88 normoglycemic patients (glucose < 140 mg/dl) served as the control group. Hyperglycemic patients (glucose ≥ 140 mg/dl) were randomized to intensive glycemic control (IGC) for almost 24 h after PCI (n=54; 80–140 mg/dl) or conventional glycemic control (CGC, n=52; 180–200 mg/dl). EPC number (CD34+/KDR+), differentiation, and SIRT1expression were assessed immediately before, 24 h, 7, 30 and 180 days after PCI.

*Results*
Hyperglycemic patients had lower EPC number and differentiation and lower SIRT1 levels than normoglycemic patients ($P < 0.01$). The EPC number, their capability to differentiate, and SIRT1 levels were significantly higher in IGC group than in CGC, peaking after 24 h ($P < 0.01$). In the IGC group, the salvage index was greater than in patients treated with CGC ($P < 0.001$).

*Conclusions*
Optimal peri-procedural tight glycemic control, by increasing EPC number and their capability to differentiate, may improve the myocardial salvage.

# Pancreatic cancer stem cells give rise to vasculogenic mimicry and secrete potential diagnostic markers

Dando Ilaria[1], Biondani Giulia[1],Dalla Pozza Elisa[1], Brandi Jessica[2], Zoratti Elisa[3], Zeeberg Katrine[4], Boschi Federico[5], Cecconi Daniela[2], Reshkin Stephan Joel[4], Scupoli Maria Teresa[3], Palmieri Marta[1]

[1]University of Verona, Department of Life and Reproduction Sciences, Verona, Italy
[2]University of Verona, Department of Biotechnology, Verona, Italy
[3]University of Verona, Applied Research on Cancer Network (ARC-NET) Verona, Italy
[4]University of Bari, Department of Biosciences, Biotechnologies and Biopharmaceutics, Bari, Italy
[5]University of Verona, Department of Computer Science, Verona, Italy

*Introduction*
Pancreatic ductal adenocarcinoma (PDAC) is generally asymptomatic until the late stage of the disease and it often metastasizes. Cellular dissemination leading to metastasis occurs prior to the formation of an identifiable primary tumour and strictly correlates with the presence of cancer stem cells (CSCs). This observation renders imperative the identification of the specific biological features of CSCs, in order to improve PDAC diagnosis and prognosis.

*Objectives*
1. Obtainment and characterization of CSCs.
2. Generation of three-dimensional (3D) culture models.
3. Secretome analysis.

*Results*
1. CSCs obtained from five out of nine PDAC cell lines showed different tumorsphere forming ability, increased expression of the stem cell markers EpCAM and CD44v6, decreased expression of the epithelial marker E-cadherin, and higher resistance to five anti-cancer drugs compared to parental cell lines. Interestingly, CSCs were able to re-differentiate into parental cells once cultured in parental growth condition, as demonstrated by re-acquisition of the epithelial features and the increased sensitivity to anti-cancer drugs. Finally, PDAC CSCs injected into nude mice developed a larger subcutaneous tumor mass and showed a higher metastatic activity compared to parental cells.
2. 3D models obtained by growing cells on Matrigel showed that only CSCs presented a tube-like structure resembling vasculogenic mimicry, a feature associated with aggressiveness. Furthermore, CSCs showed an increased expression of the endothelial markers CD31, CD34, eNOS, and CD144 compared to parental cells.
3. iTRAQ analysis identified 71 proteins secreted at higher level by CSCs than parental cells and 9 proteins secreted only by CSCs. Three of the proteins secreted only by CSCs were analysed on 21 control and 100 PDAC patient sera. The amount of two of these, ceruloplasmin and MARCKS, was significantly higher in PDAC sera.

*Conclusion*
CSCs derived from PDAC cell lines are a valuable model for a deep understanding of PDAC biology and for marker discovery.

# Increased fucosylation activity has a pivotal role in invasive and metastatic properties of head and neck cancer stem cells

Desiderio Vincenzo[3], Papagerakis Petros[2,4,5], Tirino Virginia[3], Zheng Li[2], Matossian Margarite[1], De Rosa Alfredo[6], Paino Francesca[3], Mele Luigi[1,3], Montella Roberta[3], Papagerakis Silvana[1,2], Papaccio Gianpaolo[3]

[1]Department of Otolaryngology, Medical School, University of Michigan, Ann Arbor, MI, USA
[2]Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA
[3]Department of Experimental Medicine, Section of Biotechnology and Medical Histology and Embryology, Second University of Naples, Italy
[4]Department of Orthodontics and Pediatric Dentistry, School of Dentistry, University of Michigan, Ann Arbor, MI, USA
[5]Center for Organogenesis, Medical School, University of Michigan, Ann Arbor, MI, USA
[6]Department of Head and Neck Surgery, Second University of Naples, Italy

Oral squamous cell carcinoma (OSCC) is an aggressive malignancy with high mortality rates. Major challenges for OSCC management include development of resistance to therapy and early formation of distant metastases. Cancer stem cells (CSCs) have emerged as important players in both pathologic mechanisms. Increased fucosylation activity and increased expression of fucosylated polysaccharides, such as Sialyl Lewis X (SLex), are associated with invasion and metastasis. However, the role of fucosylation in CSCs has not been elucidated yet. We used the spheroid culture technique to obtain a CSC-enriched population and compared orospheres with adherent cells. We found that orospheres expressed markers of CSCs and metastasis at higher levels, were more invasive and tumorigenic, and were more resistant to cisplatin/radiation than adherent counterparts. When we analyzed fucosyltransferase (FUT) expression, we found FUT3 and FUT6 highly up-regulated in orospheres, resulting in increased SLex production and increased stickiness in shear flow assay. Inhibition of fucosylation negatively affected orospheres formation and invasion of oral CSCs. These results demonstrate that orospheres are enriched in CSCs and that fucosylation and fucosylated saccharides, such as Sialyl Lewis X, are or paramount importance for CSC invasion. Inhibition of fucosylation may be used to block CSCs and metastatic spread.

# Locked Nucleic Acid (LNA)-miR-221 Inhibitor as promising new anti-myeloma agent

Di Martino Maria Teresa[1,2], Gallo Cantafio Maria Eugenia[1], Gullà Annamaria[1], Amodio Nicola[1], Altomare Emanuela[1], Morelli Eugenio[1], Tagliaferri Pierosandro[1,2], Tassone Pierfrancesco[1,2,3]

[1]Department of Experimental and Clinical Medicine, Magna Graecia University and Medical Oncology Unit, Catanzaro, Italy

[2]T. Campanella Cancer Center, Salvatore Venuta University Campus, Catanzaro, Italy

[3]Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, PA, USA

miR-221/222 are two highly homologous microRNAs (miRNAs), encoded in tandem on the chromosome X, whose up-regulation has been found in several malignancies. Both miRNAs are thought to promote cell proliferation via down-regulation of p27 and/or p57, two negative regulators of G1 to S phase cell cycle progression. We demonstrated up-regulation of both miRNAs in malignant plasma cells (PCs) from multiple myeloma (MM) patients belonging to distinct TC (traslocation/Cyclin) groups, including TC2 and TC4. A rising body of evidence suggests that silencing miRNAs with oncogenic potential could represent a novel approach for human cancer therapy. We previously demonstrated that silencing miR-221exerts significant anti-MM activity and triggers canonical targets in vitro and in vivo (Di Martino et al. Oncotarget, 2013). Then, in the aim to progress to clinical translation of our proof-of-principle findings, we designed and investigated the anti-tumor activity and the suitability for systemic delivery of novel and originally designed LNA-miR-221 inhibitors. Finally, we selected a 13-mer miR-221 inhibitor, named LNA-i-miR-221, which took advantage from locked nucleic acid (LNA) technology and phosphorothioate backbone for increasing the seed sequence binding stability and nuclease resistance in vivo. We demonstrated that LNA-i-miR-221 inhibited growth and survival of t(4;14) MM cells in vitro, knock-down of miR-221 levels and increased p27Kip1 mRNA and protein, a miR-221 canonical target. In vivo, systemic delivery of LNA-i-miR-221 exerted significant anti-tumor activity in MM xenografted SCID/NOD mice (Di Martino et al, PlosOne 2013). We here report the pharmacokinetics of LNA-i-miR-221 by in situ hybridization (ISH) assay in tumors and in different vital organs including liver, kidney, bone marrow and heart. After a single i.p. dose of 25mg/kg, we detected the presence of LNA-i-miR-221 from 2 up to 7 days in tumors and mouse tissues. Interestingly, any toxicity was detected with long lasting presence of the inhibitors in vital organs. To evaluate the maximum tolerated doses, we analyzed in vivo antitumor activity following dose escalation treatments ranging from 10 to 100 mg/kg delivered at day 1,4,7,14, 22, 28. All animal treatments were well tolerated. No changes in mice behavior or organ toxicity was observed in treated mice. The antitumor activity observed suggest a saturable carrier-mediated transport of the LNA-i-miR-221 inhibitors. These preliminary observations need further investigations to better explore the dynamic and the kinetics of these tiny LNA molecules in vivo. The antitumor activity observed in MM and in other tumor cell lines support the rationale for development of this novel and highly efficient LNA-miR-221 inhibitor as a promising drug in subsequent primate toxicology studies.

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# Autophagy in renal angyomiolipoma

Fiorini Claudia [1], Burato G.[1], Sanavio S.[2], Gobbo S.[2], Brunelli M.[1], Martignoni G.[2]

[1]Department of Pathology and Diagnostics, University of Verona, Italy
[2]Pederzoli Hospital, Peschiera d/G, Department of Anatomic Pathology, Peschiera d/G, Verona, Italy

*Objective*
Autophagy is a way of lysosomal self-degradation essential for cellular homeostasis, which can be used by tumor cells to survive in adverse conditions. The mTOR complex is known as an inhibitor of the autophagic process and it is described constitutively active in renal angyomiolipoma tumor. Recent studies suggested its possible autophagy activator effect by TFEB dephosphorylation.
The aim of our work is to verify the presence of autophagy and its regulatory mechanisms in renal angiomyolipoma.

*Materials and Methods*
Same cases of renal angiomyolipoma underwent to immunohistochemical studies, immunofluorescence and Western blot, to the detection of the presence of proteins that occur in autophagy and lysosomal activity, mTOR and of the transcription factor TFEB. We have also carried out a study of electron microscopy on tumor cells.

*Results*
With methods described above it was possible to demonstrate the presence of autophagy, through the expression of LC3B; the functional activation of mTOR, through the research of the phosphorylated p70S6K; and the expression of TFEB. Electron microscopy also showed the presence of several cytoplasmic organelles double membrane.

*Conclusions*
In renal angiomyolipoma: l) there is autophagy; 2) mTOR is a constantly active factor; 3) there is the TFEB expression; 4) it is possible that the autophagy presence is due to the TFEB activators effect; 5) The presence of autophagy is a rational basis for the enhancement of medical therapy in these lesions, through the combination of drugs acting on the autophagic process (chloroquin) drugs mTOR inhibitors

# Methods to be used to stratify patients for more likely response to mTOR inhibitors when testing mTOR related biomarkers in clear cell renal cell carcinoma

Fiorini Claudia[1], Massari Francesco[2], Pedron Serena[1], Sanavio Sara[1], Ciccarese Chiara[2], Porcaro Antonio Benito[3], Artibani Walter[3], Bertoldo Francesco[4], Zampini Claudia[1], Modena Alessandra[2], Sava Teodoro[2], Caliò Anna[1], Ficial Miriam[1], Chilosi Marco[1], Tortora Giampaolo[2], Scarpa Aldo[1], Martignoni Guido[5], Brunelli Matteo[1]

[1]Department of Pathology and Diagnostic, University of Verona, Italy
[2]Medical Oncology, Azienda Ospedaliera Universitaria Integrata di Verona AOUI, University of Verona, Italy
[3]Urologic Clinic, Department of Oncological and Surgical Sciences, University of Verona, Italy
[4]Department of Medicine, Università di Verona, Verona, Italy
[5]Department of Anatomic Pathology, Pederzoli Hospital, Peschiera d/G, Verona, Italy

*Objective*
mTOR inhibitors play an important role in the targeted treatment of clear cell renal cell carcinoma, however no pre-selection of patients is available to predictive high efficacy to targeted inhibitors in the metastatic setting. Different potential mechanisms may also lead to resistance to mTOR inhibitors. Evidence of activation of predictive biomarkers by molecular testing on formalin-fixed and paraffin embedded cancerous tissue blocks does also depend to different technique used and may in part justify absence of responsiveness to targeted therapies. The visualization of active mTOR pathways has not been tested and compared on the same cohort of patients by using simultaneously the most common techniques such as immunohistochemistry, Western blot and immunofluorescence analysis. Aim. We sought to investigate and compare the capability of three major techniques in evidencing active function of molecules related to mTOR pathways on renal cell carcinoma.

*Materials and Methods*
16 patients affected by clear cell renal cell carcinoma were recruited. Immunohistochemichal (IHC ), Western blot (WB) and immunofluorescence (IF) were performed by using the following molecules for active detection of mTOR pathways: mTOR, ph mTOR, p70S6k, ph p70S6k polyclonal and monoclonal, ph S6 Rb and ph 4EBP1.

*Results*
No one molecules was simultaneously revealed by all three techniques. ph p70S6k monoclonal was detected by two (IHC and IF) methods. The other molecules were detected by only one technique as follows: IHC showed the sensitivity to detecting the expression of ph mTOR (Ser 2448) and ph p70S6K (Thr389) molecules, WB was the sensitive method to detect the expression of p70-S6K molecules (Thr389), ph S6Rb and ph 4EBP1 ones (Thr37/46) and the IF was sensitive in detecting the expression of mTOR (Ser235/236 ).

*Conclusion*
1) there is a significant difference in detecting mTOR pathway's active biomarkers by using three common techniques such as immunohistochemistry, Western Blot and immunofluorescence analysis; 2) the methods to detect active molecules of the mTOR pathway is important when justifying responsiveness or resistance to targeted drugs; 3) clinical trials need an agreement for standard methods to use for tissue testing in order to improve accuracy when correlation is performed between scoring the mTOR pathway activation and clinical end-points such as efficacy to targeted drugs.

# Zn(II)-compound degrades mutant p53 through autophagy

Garufi Alessia[1,2], Avantaggiati Maria Laura[3], D'Orazi Gabriella[1,2]

[1]Regina Elena National Cancer Institute, Department of Experimental Oncology, Rome, Italy
[2]University "G. d'Annunzio", Department of Medical, Oral and Biotechnological Sciences, Chieti, Italy
[3]Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC, USA

*Objective*
TP53 oncosuppressor is frequently mutated in cancer contributing to tumor progression and resistance to therapies. Mutant p53 (mutp53) proteins are prone to loss of the Zn(II) bound to the core DNA binding domain and this in turn favours protein unfolding, aggregation and impairment of DNA binding and transcription activity. Mutp53 is often highly expressed in cancers due to its increased half-life, therefore, targeting mutp53 protein for reactivation of wild-type function(s) and/or for mutp53 degradation may have therapeutic significance.

*Materials and Methods*
Here we used a fluorescent curcumin-based Zn(II) complex [Zn(II)-curc] and assessed its ability to affect mutp53 degradation through autophagy. Biochemical and molecular biology studies were carried out.

*Results*
Zn(II)-curc restored the folded conformation of mutp53 proteins (R175H), inducing wtp53 DNA binding and transactivation. Consequently, Zn(II)-curc triggered apoptosis in mutp53-carrying cell lines. Zn(II)-curc promoted mutp53H175 degradation through autophagy. Suppression of autophagy prevented Zn(II)-curc-induced mutp53H175 degradation and restoration of wild-type p53 oncosuppressor activities. On the contrary, the proteasome inhibitor MG132 failed to do so, suggesting that autophagy was the main route for p53H175 degradation. Mechanistically, Zn(II) restored the wtp53 ability to induce the expression of the p53 target gene DRAM (damage-regulated autophagy modulator), a key regulator of autophagy, leading to autophagic induction. Accordingly, inhibition of wtp53 transactivation by pifithrin-Đ (PFT-Đ) impaired both autophagy and mutp53H175 degradation induced by Zn(II)-curc.

*Conclusions*
These results uncover a novel mechanism employed by Zn(II)-curc to reactivate mutp53H175 which involves mutp53 degradation via wtp53-mediated autophagy. We thus propose that due to its effect in reducing the levels of accumulated mutp53H175, together with the ability of ameliorating mutp53H175 misfolding, Zn(II)-curc may serve as a key lead compound for the development of anticancer drugs to effectively treat mutp53H175-carrying tumors.

# FAK silencing affects HCC development and progression both in vivo and in vitro

Gnani Daniela[1], Ceccarelli Sara[1], Artuso Simona[2], De Stefanis Cristiano[1], Leonetti Carlo[2], Alisi Anna[1]

[1]Bambino Gesù Children's Hospital, Liver Research Unit, Rome, Italy
[2]Regina Elena National Cancer, Experimental Preclinical Laboratory, Rome, Italy

*Objective*
During the multi-step process of HCC pathogenesis, the activation/inactivation of several epigenetic and signaling pathways, mainly those regulating proliferation/apoptosis balance, may play a major role. HCC displayed Focal adhesion kinase (FAK) overexpression and activation suggesting a role of this protein in tumor development and progression. The aim of this work was to study the effect of FAK silencing in an in vivo HCC xenograft NOD/SCID mouse model and in HepG2 cell lines.

*Materials and Methods*
We established HepG2Luc-siCTRL or HepG2Luc-siFAK cells that were intrahepatically injected in NOD/SCID mice to analyze in vivo tumorigenicity or used to study the role of FAK on HepG2 cell homeostasis and invasion.

*Results*
Xenograft experiments demonstrated that the injection of HepG2Luc-siFAK resulted in a tumor mass 30-40% lower than that induced by HepG2Luc-siCTRL. In HCC from silenced HepG2 clones the expression of FAK and PCNA was drastically reduced. Furthermore, FAK-silenced HepG2 cells showed a statistically significant reduced in vitro proliferation and invasion rate. The silencing of FAK also induced a reduction of phosphorylated/inactive tumor suppressor protein PTEN. Interestingly, a down-regulation of EZH2 at the transcript level was observed in FAK-silenced cells together with a decrease in the EZH2 nuclear protein levels and a reduced expression of the EZH2 specific 3meH3k27 activity. We also reported an increased expression of the EZH2-targeted mir-200b compared to control cells. This FAK-mediated EZH2 down-regulation seems to be mir-101 independent, since no significant changes were reported.

*Conclusion*
In conclusion our results showed that FAK silencing can counteract the invasive and high proliferative cancer phenotype in HCC. Moreover, the silencing critically affect the activity of EZH2 suggesting a potential interaction between FAK and EZH2. However, further studies are needed to shed light on FAK and EZH2 molecular cross-talk and to elucidate the mechanisms of FAK-mediated proliferation during HCC progression.

# Onconase induces autophagy sensitizing pancreatic cancer cells to gemcitabine and activates Akt/mTOR pathway in a ROS-dependent manner

Gotte Giovanni[1], Fiorini Claudia[1], Cordani Marco[1], Picone Delia[2], Donadelli Massimo[1]

[1]Department of Life & Reproduction Sciences, Biochemistry Section, University of Verona, Italy
[2]University of Naples "Federico II", Department of Chemical Sciences, Naples, Italy

Onconase® (ONC) is a member of RNase super-family secreted in oocytes and early embryos of Rana Pipiens. In the last years, the research interest about this small and basic frog RNase, also called ranpirnase, constantly increased for its high cytotoxicity and anticancer properties. Onconase is currently used in clinical trials for cancer therapy, however the precise mechanisms determining cytotoxicity in cancer cells have not been fully investigated yet.

In this work, we evaluate the anti-tumoral property of onconase in pancreatic adenocarcinoma cells and in non-tumorigenic cells as a control. We demonstrate that ONC stimulates a strong anti-proliferative and pro-apoptotic effect in cancer cells reporting for the first time that ONC triggers Beclin1-mediated autophagic cancer cell death. In addition, we demonstrate that ONC inhibits the expression of mitochondrial uncoupling protein 2 (UCP2) and of manganese-dependent superoxide dismutase (MnSOD) triggering mitochondrial superoxide ion production. Reactive oxygen species (ROS) induced by ONC are responsible for Akt/mTOR pathway stimulation determining sensitivity of cancer cells to mTOR inhibitors and lessening autophagic stimulation.

This indicates that ROS/Akt/mTOR axis is a strategy adopted by cancer cells to reduce ONC-mediated cytotoxic autophagy stimulation. In addition, we show that ONC can sensitize pancreatic cancer cells to the standard chemotherapeutic agent gemcitabine allowing a reduction of drug concentration when used in combination setting, suggesting a lowering of chemotherapy-related side effects.

Altogether, our results shed more light on the mechanisms lying at the basis of ONC antiproliferative effect in cancer cells and support its potential use to develop new anticancer strategies.

# Anti-inflammation action of arachidonoyl amide derivatives in a human keratinocytes cell line model of acute inflammation

Gregorelli Alex[1], De Rosa Margherita[2], Soriente Annunziata[2], Saturnino Carmela[3], Menegazzi Marta[1]

[1]Department of Life and Reproduction Sciences, Section of Biochemistry, Verona, Italy
[2]University of Salerno, Department of Chemistry and Biology, Salerno, Italy
[3]University of Salerno, Department of Pharmacy, Salerno, Italy

*Objective*

Arachidonic acid (AA) is a poly unsaturated fat of the cell membrane phospholipids and it is released by phospholipase A2 after stimulation. AA is the substrate of cyclooxygenase-1 and 2 producing prostaglandins and prostacyclins that are well known inflammatory mediators. However, the effect of AA on inflammation is rather contradictory: in fact, some papers report its specific anti-inflammatory effect. Since AA is not easy to be used for its instability and light sensitivity, we synthesized seven new arachidonoyl amide derivatives (AA-Ds).

*Materials and Methods*

Human keratinocyte cells (HaCaT) were induced with TNFÐ and IFNγ. Gene expression levels were measured by real time PCR. Western blot experiments were performed to measure total or phosphorylated protein levels. Transcription factors activity were tested by EMSA.

*Results*

We demonstrate the anti-inflammatory action of AA-Ds since AA-Ds pre-treated cells showed a strong decrease in iNOS mRNA levels with respect to cytokines-induced cells. In addition, TNFα, Chemokine C-X-C motif ligand 9 and C-X-C motif ligand 10 gene expression were down-regulated. EMSA results have allowed us to exclude the involvement of signal transducers and activators of transcription-1 (STAT1) and nuclear factor-kappa B (NF-kB) canonical pathways in the down regulation of these genes expression. The possible shortening effect on iNOS mRNA half-life was also excluded by using the transcriptional inhibitor Actinomycin D. Recently results show some differences in the expression of components of AP-1 heterodimer. AA-Ds pre-treated samples show the highly increase Fra-1, c-Jun and c-Fos mRNA levels.

*Conclusion*

These results could lead up to develop new molecules against inflammatory processes which should be able to break off cytokines-induced signal transduction.

# Doxorubicin metronomic chemotherapy overcome drug resistance by targeting cancer cell mitochondrial metabolism

Kopecka Joanna, Gazzano Elena, Gulino Giulia Rossana, Volante Marco, Ghigo Dario, Riganti Chiara

University of Torino, Department of Oncology, Torino, Italy

*Objective*

The conventional protocols of chemotherapy are usually based on the maximum tolerated dose of the chemotherapeutic drug: despite the good therapeutic efficacy, this approach is often associated with several side effects and does not prevent the development of drug resistance. Alternative approaches (the so called "metronomic chemotherapy") are based on the administration of repeated low doses and/or on the continuous prolonged infusion of low doses of chemotherapeutic drugs. Metronomic chemotherapy shows several advantages in terms of pharmacokinetic and pharmacodynamic effects versus standard chemotherapy protocols. Its efficacy against multidrug resistant cells overexpressing the efflux transporter P-glycoprotein (Pgp) has been poorly investigated.

*Materials and Methods*

We compared the effects of two repeated low doses versus a single high dose of doxorubicin in drug sensitive Pgp-negative and drug resistant Pgp-positive human and murine cancer cells in vitro and in vivo.

*Results*

Metronomic chemotherapy with doxorubicin was significantly ($p < 0.05$) more cytotoxic in vitro and in vivo against drug resistant tumors than conventional treatment with single high dose. The greater efficacy of the repeated low doses treatment was due to the increased level of intracellular reactive oxygen species, produced by the higher electron flux from complex I to complex III of the mitochondrial respiratory chain. This process induced mitochondrial oxidative damage, decrease in mitochondrial ATP, loss of mitochondrial potential and resulted in drug resistant tumors death.

*Conclusions*

We demonstrated that, changing timing and doses of administration, a chemotherapeutic agent may become effective in drug resistant tumor cells, by targeting their mitochondrial redox metabolism. We propose that metronomic chemotherapy based on repeated low doses of doxorubicin is worthy of clinical investigations against drug resistant Pgp-expressing tumors.

# Methylation and epigenetic modification by 5' azacytidine and acid valproic treatment increase stemness characteristics in bone sarcoma cell lines

La Noce Marcella, Tirino Virginia, Paino Francesca, Desiderio Vincenzo, Papaccio Gianpaolo

Second University of Naples, Department of Experimental Medicine, Naples, Italy

*Objective*

Bone sarcoma is an aggressive malignancy with high mortality rate. Despite recent advances, the prognosis is still extremely poor. Bone sarcomas contain a small cell population with stem cell like properties, referred to as cancer stem cells characterized by CD133 expression (Tirino et al, 2009;2011). The biological relevance and regulatory mechanism of CD133 expression are not yet understood. Aim of this study is to elucidate mechanisms regulating aberrant expression of CD133 and stemness phenotype.

*Materials and Methods*

Saos-2, MG63 and BS15 cell lines were treated with 0,5 mM valproic acid (VPA) and 3μM 5'azacytidine (5-AZA) for 48 hours alone, and in combination. CD133 expression as well as stemness markers expression including OCT4, Sox2 and Nanog was analyzed by flow cytometry and real-time PCR. Vimentin and osteocalcin levels were also tested. Sarcospheres formation rate was assessed as spheres number/seed single cells number.

*Results*

After treatment with 5-AZA or VPA, the expression level of CD133 mRNA as well as of protein was significantly increased in all three cell lines. Also OCT4, Sox2 and Nanog, stemness markers, and vimentin, mesenchymal marker resulted to be up-regulated after treatment by real time-PCR. On the contrary, the expression level of osteocalcin remained similar before and after treatment. Interestingly, combined treatment with 5-AZA and VPA induced an increase of CD133 expression in a synergistic manner in all three cell lines. In addition, sarcospheres formation rate was increased after drugs treatment compared to untreated cells. And also in this case, the drug combination lead to synergistic increase of formation rate of spheres.

*Conclusion*

In conclusion, our results indicate that DNA methylation is an important determinant of CD133 and stemness profile in human bone sarcomas and this mechanism may be associated with histone deacetilase inhibition.

# The TrkAIII oncoprotein inhibits mitochondrial free radical ROS-induced death of SH-SY5Y Neuroblastoma cells by augmenting SOD2 expression and activity at the mitochondria, within the context of a tumour stem cell-like phenotype

Di Ianni Natalia, Ruggeri Pierdomenico, Farina Antonietta, Cappabianca Lucia, Ragone Marzia, Ianni Giulia, Gulino Alberto and Mackay Andrew

Department of Applied Clinical and Biotechnological Sciences, University of L'Aquila, Italy

The developmental and stress-regulated alternative TrkAIII splice variant of the NGF receptor TrkA is expressed by advanced stage human neuroblastomas (NBs), correlates with worse outcome in high TrkA expressing unfavourable tumours and exhibits oncogenic activity in NB models.

*Objective*
To determine the effect of TrkAIII upon Superoxide dismutase expression, mitochondrial ROS accumulation, ROS-mediated cell death death and staminality in human SH-SY5Y neuroblastoma cells.

*Materials and Methods*
Stable cell transfection; mitochondrial ROS accumulation and cell death assays; mitochondrial purification and H2O2 production assay; Superoxide dismutase zymography; siRNA knockdown; TrkA kinase inhibitor studies (GW441756, K252a, CEP-701 and Gö6976); RT-PCR; Western blotting; Tumour spheroid growth assays.

*Results*
Constitutive TrkAIII expression in human SH-SY5Y neuroblastoma cells inhibits Rotenone, Paraquat and LY83583-induced mitochondrial free radical reactive oxygen species (ROS)-mediated death by stimulating SOD2 expression, increasing mitochondrial SOD2 activity and attenuating mitochondrial free radical ROS production, in association with increased mitochondrial capacity to produce H2O2, within the context of a more tumour stem cell-like phenotype. This effect is reversed by the specific TrkA tyrosine kinase inhibitor GW441756, by the multi-kinase TrkA inhibitors K252a, CEP-701 and Gö6976, which inhibit SOD2 expression, and by siRNA knockdown of SOD2 expression, which restores the sensitivity of TrkAIII expressing SH-SY5Y cells to Rotenone, Paraquat and LY83583-induced mitochondrial free radical ROS production and ROS-mediated death.

*Conclusion*
The data implicate the novel TrkAIII/SOD2 axis in promoting NB cell staminality and resistance to mitochondrial free radical-mediated death, and suggest that the combined use of TrkAIII and/or SOD2 inhibitors together with agents that induce mitochondrial free radical ROS-mediated death could provide a therapeutic advantage that may also target the stem cell niche in high TrkA expressing unfavourable neuroblastoma.

# Effects of two novel quinoline-based non-nucleoside DNA methyltransferase inhibitors in osteosarcoma and Ewing sarcoma cells

Manara Maria Cristina[1], Valente Sergio[2], Cristalli Camilla[1], Baricordi Cristina[1], Zwergel Clemens[2], Gros Christine[3], Aussagues Yannick[3], Gregoire Jean-Marc[3], Ausseil Fréderic[3], Ruoppolo Giovanni[4], Arimondo Paola B.[3], Picci Piero[1], Mai Antonello[2,5], Scotlandi Katia[1]

[1]Rizzoli, Orthopaedic Institute, Experimental Oncology Laboratory, Bologna, Italy
[2]Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, Italy
[3]USR3388 CNRS-Pierre FabreETaC, CRDPF, 3 avenue H. Curien, Toulouse, France
[4]Dipartimento di Organi di Senso, Sapienza Università di Roma, Italy
[5]Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza Università di Roma, Italy

*Objective*
Identification of new drugs against sarcomas still represents an urgent clinical need due to the rarity of these diseases. Aberrant DNA methylation of gene promoter regions is recognized to be highly involved in cancer and efforts have been made to synthesize non-nucleoside compounds that can effectively modulate gene expression, with bearable side effects. Two novel DNMT inhibitors (DNMTi), MC3353 and MC3343, were tested in Ewing sarcoma (EWS) and osteosarcoma (OS) cell lines, to determine their effects on proliferation, apoptosis/necrosis induction, and cell differentiation.

*Materials and Methods*
In vitro antitumor activity was evaluated in a panel of ES and OS cell lines. Specificity of DNMT inhibition was verified by western blot and immunofluorescence, while total DNMT activity was measured by using EpiQuik DNA Methyltransferase Activity assay. Effects on neural differentiation was assessed by β-III tubulin and heavy neurofilament modulation. Effects on osteoblastic differentiation was carried out on Saos-2 and evaluated by RT-PCR.

*Results*
As a DNMTi, MC3353 showed reduced efficacy compared to MC3343, but re-induced gene expression in a cellular luciferase CMV reporter system. In vitro, MC3343 was found to slow cell proliferation by increasing the percentage of cells in G1 or G2/M phases, while MC3353 compound was unable to modulate cell cycle but induced a significant increase in cell death indicating a cytotoxic rather than a cytostatic effect. Modulation of key regulators of cell cycle and evaluation of PARP cleavage confirmed these results. Both DNMTi modulated the expression of neural markers in EWS cells. However a significant increase in neurite outgrowth was observed only after MC3343 treatment, concurrently with inhibition of proliferation. In OS cells DNMTi increased both matrix mineralization and expression of genes specifically related to osteoblastogenesis.

*Conclusion*
This is one of the first study on non-nucleoside DNMTi in sarcomas, opening new therapeutic possibilities in patient management.

# Insulin-like growth factor II (IGF-II) mRNA binding protein 3 expression predicts poor prognosis in primary Ewing sarcoma patients

Mancarella Caterina[1], Calzolari Linda[1], Grilli Andrea[1], Ferrari Stefano[2], Donati Davide[3], Scotlandi Katia[1]

[1]CRS Development of Biomolecular Therapies, Experimental Oncology Lab, Rizzoli Institute, Bologna, Italy
[2]Pathology Section, Musculoskeletal Tumours Chemotherapy Section, Rizzoli Institute, Bologna, Italy
[3]III Orthopaedic Clinic, Rizzoli Institute, Bologna, Italy

*Introduction*
Insulin-like growth factor II (IGF-II) mRNA binding protein 3 (IMP3) belongs to an oncofetal protein family which binds RNAs and influences their transcript target fate. In vitro studies demonstrated that IMP3 is de novo synthesized in malignancies where promotes cell growth, proliferation and metastases. Moreover, IMP3 is emerging as a promising indicator of outcome in several cancers. Ewing sarcoma (EWS) is a rare bone and soft tissue tumor where the IGF system plays a pivotal role in cell growth. Aim of this study was to verify the value of IMP3 as biomarker of EWS progression.

*Material and Methods*
Total RNA was extracted from two independent cohorts of 30 and 109 snap-frozen tissues from localized primary EWS samples with more than 5 years of follow-up. Gene expression profile of IMP3 was analysed by Affymetrix platform in 30 patients or by qRT-PCR (Applied Biosystem) in 109 cases to obtain a technical and qualitative validation of the results. Human normal stromal stem cells were used as calibrator. Log rank ratio and Kaplan-Meier survival curves were used as statistical methods.

*Results*
Gene expression analysis of IMP3 by microarray suggested a relevance for IMP3 as prognostic marker ($p= 0.047$). This was confirmed in the series of 109 patients: log-rank tests showed an association between higher IMP3 expression and worse overall survival both dividing patients according to mediane value ($p=0.049$) or to 33° and 66° percentile ($p= 0.017$). No statistical significant association between IMP3 expression and other clinicopathological parameters was evidenced.

*Conclusions*
In this study we describe for the first time the prognostic relevance of IMP3 expression as a predictor of outcome in EWS. High expression of IMP3 is significantly associated with a worse overall survival and its detection may be used to stratify patients with different risk of progression at the time of diagnosis.

# Inhibition of Hsp60 expression by doxorubicin and replicative senescence instauration in mucoepidermoid carcinoma cells

Marino Gammazza Antonella[1,2], Caruso Bavisotto Celeste[1,2], Barone Rosario[1,2], Campanella Claudia[1,2], Gorska Magdalena[3], Cappello Francesco[1,2], Guercio Annalisa[4], Zummo Giovanni[1], Di Felice Valentina[1]

[1]University of Palermo, Department of Experimental Biomedicine and Clinical Neurosciences, Palermo, Italy
[2]Euro-Mediterranean Institute of Science and Technology, Palermo, Italy
[3]Medical University of Gdansk, Department of Medical Chemistry, Gdansk, Poland
[4]Istituto Zooprofilattico Sperimentale della Sicilia, Palermo, Italy

*Objective*

The resistance of cancerous cells to chemotherapy is the main limitation for cancer treatment. Since cancer cells proliferate indefinitely, a requisite for their immortalization is to bypass replicative senescence (RS). RS is a natural barrier used by cells against tumorigenesis and some key components of senescence process may be regulated by changes in chaperones levels. Doxorubicin (DOX) is an anthracycline antibiotic used in cancer chemotherapy with inhibiting effect on some molecular chaperones. This work is to determine the effect of DOX on the expression levels of the chaperonin Hsp60 in a lung carcinoma cell line (NCI-H292) to reveal a new anticancer mechanism.

*Materials and Methods*

NCI-H292 cell were treated with DOX (20nM, 40nM, 80nM, 160nM) for 5 days. To determine the effect of DOX on RS, selected markers of senescence and cell cycle analysis were conducted. Moreover, Hsp60 expression level was evaluated using qPCR and western blot. Hsp60 hyperacetylation and Hsp60 ubiquitination was investigated using immunoprecipitation.

RESULTS: A dose-dependent reduction of cell viability was observed. Cells exhibited typical senescent phenotype, positivity for SA β-gal assay and showed cytoskeleton remodelling due to an increase of vimentin level at 40nM, 80nM, 160nM. Moreover, cell cycle analysis showed that the cells were in the G0/G1 phase at 80nM, 160nM. The treatment increased the expression mRNA, reduced the protein level and induced hyperacetylation and ubiquitination of Hsp60.

*Conclusion*

DOX treatment resulted in cellular senescence, which seems to coincide with decreased level and hyperacetylation of Hsp60. This reduction could be due to its ubiquitination. Hsp60 is involved in tumor-invasiveness. The deregulation of Hsp60 activity, could affect protein folding and cause proteotoxic stress ultimately resulting in the switch of pro-survival signaling to cancer cell senescence. Finally, DOX could interfere with the pro-tumoral role of Hsp60 which could be used as target for chemotherapeutic treatment.

# Circulating HIF-1alpha expression as a predictive biomarker of bevacizumab efficacy in metastatic colorectal cancer patients

Marisi Giorgia[1], Ulivi Paola[1], Scarpi Emanuela[2], Passardi Alessandro[3], Nanni Oriana[2], Ragazzini Angela[2], Frassineti Giovanni Luca[3], Valgiusti Martina[3], Zoli Wainer[1], Amadori Dino[3]

[1]Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy
[2]Biostatistics and Clinical Trials Unit, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS Meldola, Italy
[3]Department of Medical Oncology, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy

*Objective*
As some controversies exist regarding the efficacy of bevacizumab (B) for the treatment of metastatic colorectal cancer (mCRC), novel predictive biomarkers are needed to improve patient selection and optimize the use of this agent. We analyzed the potential usefulness of five circulating biomarkers as predictors of B efficacy and in monitoring the disease.

*Materials and Methods*
Peripheral blood samples collected at different times (baseline, first clinical evaluation and disease progression) were available for 62 of the 376 patients enrolled in the prospective multicentric ITACa trial (NCT01878422) and randomized to receive FOLFOX4/FOLFIRI (CT) with or without B. Thirty-one of the 62 patients received CT plus B and 31 received CT only. All patients were evaluated for response according to RECIST criteria. mRNA was isolated and used for gene expression analysis of HIF-1α, COX2, VEGF-A, EPHB4 and eNOS by a quantitative RT-PCR method. Baseline expression levels of the markers and their modulation during therapy were correlated with objective response (OR) (median test), progression-free (PFS) and overall survival (OS) (Cox proportional hazards model). Potential predictive markers were identified using a treatment-by-marker interaction term in the Cox model.

*Results*
Non-responders (stable and progressive disease) had a median baseline HIF-1α expression significantly higher than responders (complete and partial response) (1.70 vs 0.94; IQR 1.05-2.32 vs 0.70-1.12) (p=0.016) in the B-treated group only. In univariate analysis, the high HIF-1α levels of this group were significantly associated with a shorter PFS (HR=1.96, 95% CI 1.17-3.30) (p=0.011) but not with OS. The HIF-1α-treatment interaction was p=0.088. No significant association was found between the other 4 genes and OR, PFS or OS. The results obtained on the modulation of gene expression during treatment are currently being analyzed.

*Conclusion*
Our preliminary data suggest that high pre-treatment HIF-1α expression levels could represent a predictive biomarker of resistance to bevacizumab.

# A redox trojan mechanism for fighting cancer

Martinotti Simona, Ranzato Elia

University of Piemonte Orientale, DiSIT- Dipartimento di Scienze e Innovazione Tecnologica, Alessandria, Italy

*Objective*

Malignant mesothelioma (MMe) is a lethal tumor arising from the mesothelium of serous cavities.
Ascorbate since long time is considered as a remedy in the treatment of cancer.
We have demonstrated that ascorbate is selectively cytotoxic toward MMe cells, showing that ascorbate-induced extracellular H2O2 production induces a strong oxidative stress in MMe cells due to their high superoxide production rate.
Hence, we have attempted at finding partner compounds that could act synergistically with ascorbate against MMe cell growth.

*Materials and Methods*

Isobologram analyses, based on in vitro cytotoxicity tests, have been utilized to find synergistic interactions of ascorbate with other compounds.
We have then focused on the mechanism of action, showing the induction of apoptosis and cell cycle arrest through measurements of caspase 3, intracellular Ca2+ level, ROS production, and gene expression signatures.

*Results*

Gemcitabine, a chemotherapeutic drug, and epigallocatechin-3-gallate (EGCG), a natural anti-cancer compound present in green tea, highlighted a synergistic behavior when mixed with ascorbate.
So we have investigated and defined a novel mechanism of action for EGCG that involves the induction of T-channel opening by H2O2, followed by [Ca2+]i homeostasis impairment, induction of intracellular ROS and eventually cell apoptosis or necrosis. These findings suggest the possible use of EGCG for MMe, and indicate T-type Ca2+ channels as a novel therapeutic target.
We have also defined a mechanism of action of the mixture of ascorbate, EGCG and gemcitabine that involves [Ca2+]i homeostasis impairment, upregulation of DAPK2, the possible deactivation of NF-ÐB, and a block of cell cycle.

*Conclusion*

We have put forward the idea of combining active nutrients and pharmaceutical drugs in the treatment of MMe. The complex of in vitro and in vivo data indicates that the mixture is synergistic in inducing cell cycle deregulation and non-inflammatory apoptosis, suggesting its possible use in MMe treatment.

# Diatomite nanoparticles for siRNA delivery in cancer cells

Martucci Nicola Massimiliano[1], Migliaccio Nunzia[1], Ruggiero Imma[1], Rea Ilaria[2], Terracciano Monica[2], Tatè Rosarita[3], De Stefano Luca[2], Rendina Ivo[2], Arcari Paolo[1], Lamberti Annalisa[1]

[1]University of Naples Federico II, Department of Molecular Medicine and Medical Biotechnology, Naples, Italy
[2]Institute for Microelectronics and Microsystems, National Council of Research, Naples, Italy
[3]Institute of Genetics and Biophysics, National Council of Research, Naples, Italy

*Objective*
Diatomite is a natural porous biomaterial of sedimentary origin, formed by fragments of diatom siliceous skeletons, called "frustules", mainly constituted by amorphous silica and characterized by a specific surface area up to 200 m2/g. Due to large availability in many areas of the world, chemical stability, and non-toxicity, these fossil structures have been widespread used in lot of industrial applications, such as food production, water extracting agent, production of cosmetics and pharmaceutics. In the present study, we have obtained nanoparticles of about 450 nm in diameter from diatomite frustules and tested them for small interfering ribonucleic acid (siRNA) transport inside human cancer cells.

*Materials and Methods*
Natural diatomite was received from DEREF (Italy). Nanometric porous particles (average size lower than 450 nm) are obtained by mechanical crushing, sonication, and filtering of micrometric frustules. The particles were then treated in hot acid solutions (H2SO4 and HCl) to remove organic and metallic impurities. Amino-modification of diatomite nanoparticles surface was performed in APTES anhydrous ethanol solution whereas an etherobifunctional linker, sulfo-GMBS, was conjugated to amino-modified diatomite surface to allow the loading of a peptide-siRNA complex. Surface modification was monitored by Fourier transformed infrared (FTIR) microspectroscopy. Human lung epidermoid carcinoma cells (H1355) were treated with diatomite nanoparticles loaded with a fluorescent siRNA and analyzed by confocal fluorescence microscope, with the appropriate filters.

*Results*
In-vitro experiments show very low toxicity on exposure of the cells to diatomite nanoparticles concentration up to 300 µg/ml for 72 h. Confocal microscopy imaging performed on cancer cells incubated with siRNA conjugated nanoparticles demonstrates a cytoplasmatic localization of vectors.

*Conclusion*
Our studies endorse diatomite nanoparticles as non-toxic nanocarriers for siRNA transport in cancer cells. Moreover, it would be expected that compared to other nanocarriers, their selective targeted functionalization will improve the delivery of anti-tumoral molecules to specific cell population.

# Nanocages for Self-triggered Nuclear Delivery of Doxorubicin at Cancer Cells

Mazzucchelli Serena[1], Bellini Michela[2], Galbiati Elisabetta[2], Fiandra Luisa[1], Truffi Marta[3], Sorrentino Luca[1], Tortora Paolo[2], Prosperi Davide[2], Corsi Fabio[1]

[1]"L. Sacco" University Hospital, Nanomedicine Laboratory, Milan, Italy
[2]University of Milan-Bicocca, NanoBioLab, Department of Biotechnology and Biosciences, Milan, Italy
[3]University of Milan, Department of Biomedical and Clinical Sciences "L. Sacco", Milan, Italy

*Objective*
Development of engineered apoferritin nanoparticle (HFn) to achieve a cumulative self-triggered nuclear delivery of doxorubicin (DOX) in cancer cells (CC) with subcellular precision.

*Materials and methods*
HFns, produced in E. coli, have been purified by heat-shock and ion-exchange chromatography, and characterized by TEM. DOX loaded by disassembly method has been quantified by fluorescence. HFn binding to HeLa cells has been assessed by flow cytometry; internalization and nuclear translocation was assessed by confocal microscopy while MTT and cell death assays are used to evaluate the effect of HFns.

*Results*
24-mer of human ferritin heavy chain was produced with high yield in E. coli, obtaining a monodisperse population of 12 nm and were efficiently loaded with DOX. HFn specifically recognized HeLa CC through transferrin receptor 1 (TfR1) binding and were internalized within 1 hour of incubation. Nanoformulated DOX showed increased nuclear delivery, antiproliferative effect, cell death and DNA damage with respect to free DOX. Also, we have proved that DOX-loaded HFn acts as a "Trojan Horse": HFn were internalized in CC more efficiently compared to free DOX, then promptly translocated into the nucleus following to the DNA damage caused by the partial release in the cytoplasm of encapsulated DOX. This self-triggered translocation allowed the drug release directly in the nuclear compartment. Finally, nanoformulated DOX displays increased efficacy towards DOX-resistant MDA-MB-468 cells.

*Conclusion*
Here, we have developed HFn nanoparticles that displays two important advantages over conventional nanocarriers of DNA-intercalating drugs: 1) the drug is principally released at its final destination with a subcellular precision through a self-triggered mechanism, thus optimizing the cytotoxic effect of the drug, and 2) the self-assembling recombinant nanocage exhibits a well documented target selectivity toward a broad selection of cancer cell types. Moreover, HFn was shown to significantly improve the accumulation of DOX in drug-resistant cancer cells.

# SADDAN-FGFR3 causes cytoskeleton disorganization and paxillin hyperphosphorylation by Src

Montone Rosa[1], Baruzzi Anna[2], Romanelli M.G.[1], Liboi Elio[1], Lievens P. M-J[1]

[1]Department of Life and Reproduction Sciences, University of Verona Medical School, Verona, Italy
[2]Department of Pathology and Diagnostics, University of Verona Medical School, Verona, Italy

*Background*
Mutations in the fibroblast growth factor receptor 3 (FGFR3) gene cause chondrodysplasias. FGFR3 is a tyrosine kinase (TK) receptor playing a key role in skeletal development. In this study, we analyzed mutations of FGFR3 associated with Severe Achondroplasia with Developmental Delay and Acanthosis Nigricans (SADDAN) and with Thanatophoric Dysplasia type II (TDII), carrying the K650M and K650E substitutions, respectively. Both substitutions affect the TK-domain functions, resulting in a strong ligand-independent constitutive FGFR3 activation. The highly phosphorylated SADDAN and TDII receptors fail to reach full maturation and accumulate in their immature isoforms in the endoplasmic reticulum, from where they induce abnormal signalling.

*Objective*
This study aimed to investigate whether the SADDAN-FGFR3 signalling could affect cytoskeletal organization through paxillin phosphorylation. Paxillin is a focal adhesion-associated protein playing an important role in cytoskeletal organization, cell morphology regulation, migration and proliferation. Paxillin is phosphorylated at Tyr118 by FAK and Src proteins.
Methods: Paxillin phosphorylation was analyzed in HEK293 cells expressing FGFR3 mutants receptors by immunoprecipitation with anti-paxillin antibodies and immunoblotting with anti-phospho-paxillin (Tyr118) antibodies. Cytoskeletal changes and paxillin localization were analyzed in HeLa cells by immunofluorescence.

*Results*
SADDAN-FGFR3 enhances paxillin phosphorylation at Tyr118 causing cell morphology alterations, and partially colocalizes with phosphorylated paxillin. The SADDAN-KD mutant, lacking kinase activity, does not affect paxillin phosphorylation, indicating the requirement of receptor enzymatic activity. Conversely, the TDII-FGFR3 mutant, although highly auto-phosphorylated, does not affect paxillin phosphorylation. Interestingly, PLC-Ð1 plays a key role in paxillin hyperphosphorylation since the SADDAN-Y754F double mutant, abolishing the binding to PLC-Ð1, does not enhance paxillin phosphorylation. Finally, the Src kinases inhibitor PP2 downregulates paxillin hyperphosphorylation, suggesting a role for Src in paxillin phospho-alterations.

*Conclusions*
Paxillin is recruited by SADDAN-FGFR3 and hyperphosphorylated through Src kinases. The results of this study will contribute to clarify the molecular events leading to actin cytoskeletal disorganization by SADDAN-FGFR3.

# Role of SPARC and MIR-29B1 in molecular effects induced by win in osteosarcoma MG63 cells

Notaro Antonietta, Sabella Selenia, Fiasconaro Giuseppe, Calvaruso Giuseppe, Giuliano Michela

Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF)
University of Palermo, Palermo, Italy

SPARC (Secreted protein acidic and rich in cysteine) is considered as a prototype of matricellular protein due to its structure and the function that it displays in regulating cell/extracellular microenvironment interactions during development and in response to injury. Earlier studies underlined pleiotropic effects of intracellular SPARC on cancer growth and, in some cancer cell lines, identified it as a tumor suppressor protein.

*Objective*
This study aimed to evaluate the role of SPARC and its related miRNA in the molecular effects induced by the cannabinoid WIN in osteosarcoma MG63 cells. In these cells WIN is not able to induce cell death but sensitizes cells to TRAIL-mediated apoptotic extrinsic pathway.

*Methods*
Western blotting analysis and qRT-PCR were employed to evaluate the expression level of SPARC and miR-29b1. Wound healing assay and zymography were employed to evaluate the migratory ability of cells.

*Results and Conclusions*
In MG63 cells WIN (5 μM) induces a dose- and time-dependent increase in the level of SPARC. Immunoprecipitation experiments showed that SPARC links caspase-8 and is responsible for its translocation and activation on plasma membrane. In this way, SPARC sensitizes osteosarcoma cells to WIN/TRAIL cytotoxic effects, as supported by SPARC silencing experiments. Moreover, we demonstrated that SPARC is not involved in migratory ability of MG63 cells, thus indicating that SPARC has a specific intracellular action as tumor suppressor. The analysis of miR-29 family cluster demonstrated that WIN induces a 300-fold increase of miR-29b1, the specific regulator of SPARC expression. Although a canonical relationship between miR-29b and SPARC can be excluded, the miR-29b1 overexpression seems, indeed, to be involved in the blocking of WIN-dependent cell migration, as demonstrated in transfected cells stably overexpressing miR-29b1. Studies are in progress to identify the cellular targets and mechanism of action of this miRNA.

# cAMP-dependent protein kinase controls a metabolic switch essential for cancer cell resistance to glucose deprivation

Palorini Roberta[1,2], Votta Giuseppina[1,2], De Palma Sara[1,3,4], Airoldi Cristina[1,2], Cirulli Claudia[1,2], Vasso Michele[1,3,4], Gelfi Cecilia[1,3,4], Alberghina Lilia[1,2], Chiaradonna Ferdinando[1,2]

[1]SysBio Centre of Systems Biology, Milano, Italy
[2]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milano, Italy
[3]Department of Biomedical Sciences for Health, University of Milano, Segrate (MI), Italy
[4]Institute of Bioimaging and Molecular Physiology, National Research Council, Segrate (MI), Italy

*Objective*
Some cancer cells usually show a strong "glucose addiction" in such a way that sugar deprivation reduce their proliferation and viability. However some cancer cells can survive without glucose within the tumor microenvironment, and hence become the most aggressive cells of a tumor. It is not well established why there is this differential response to glucose deprivation among cancer cells. Nonetheless, we believe this difference in the sensitivity to glucose deprivation may be important in the development of novel therapeutic treatments. In this study we have searched possible mechanisms leading to this "resistant" phenotype.

*Materials and Methods*
While analyzing the gene expression profiling of NCI60 cell lines in order to correlate Ras activation, often involved in glucose addiction, with other deregulated pathways, we identified a significant gene deregulation for cAMP/PKA pathway. Considering that, we treated different k-ras-transformed cancer cell lines with forskolin (FSK), hence stimulating cAMP/PKA pathway and following the effects in low glucose. Since the cAMP/PKA pathway can regulate several processes, we have investigated its role also performing different levels of "omic" analyses -transcriptomic, proteomic and metabolomic- in cells grown in low glucose and treated with FSK.

*Results*
While we observed that FSK induced prolonged cancer cells survival under glucose deprivation, the "omic" data identified a FSK-dependent tuning of glutamine metabolism. In association, the inhibition of specific enzymes like glutaminase counteracted the protective role of FSK, confirming the role of glutamine and its modulation by PKA in cell survival in absence of glucose. Accordingly, exogenous cAMP/PKA pathway stimulation led also to respiratory chain activation, intracellular ATP levels increase and ROS decrease.

*Conclusion*
Altogether these findings delineate an important role of cAMP/PKA pathway in the resistance of cancer cell to glucose starvation and suggest the pathway as possible target for cancer therapy.

# Cell viability and culture population: Statistical analysis of precision of Trypan Blue assay

Piccinini Filippo[1], Angeli Davide[2], De Santis Ilaria[2], Tesei Anna[3], Arienti Chiara[3], Bevilacqua Alessandro[1,4]

[1]Advanced Research Center on Electronic Systems (ARCES), University of Bologna, Bologna, Italy
[2]School of Biology, Department of Biological, Geological and Environmental Sciences, University of Bologna, Italy
[3]IRCCS-Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), Biosciences Laboratory, Meldola (FC), Italy
[4]School of Engineering and Architecture, Department of Computer Science and Engineering (DISI), University of Bologna, Italy

*Objective*
Cell number and viability are amongst the most spread measurements accomplished in cell biology. In particular, they are at the basis of cell proliferation and toxicity studies. Many methods exist to estimate cell viability, whilst counting the cells. However, the first method proposed, Trypan Blue (TB) dye exclusion assay, still is the most used. Nevertheless, in literature there are not any statistical studies reporting reliability analysis of the method. In this work, we performed statistical intra- and inter-rater variability experiments to validate TB assay when used to estimate cell viability and population.

*Materials and Methods*
We prepared 8 flasks containing A549 cells, a commercial cell line derived from a primary lung cancer. To perform the counts we used haemocytometers (Kova slides, Hycor Biomedical Inc.) and a commercially available TB preparation (TB solution 0.4%, SIGMA-ALDRICH). In particular, 2 expert biologists counted separately a set of 5 samples for each flask, for a total amount of 80 samples analysed. Finally, we computed the percentage and the total number of living cells for each set of samples considered, and the absolute percentage error E% between the counts of the operators.

*Results*
For cell viability, the average standard deviation values were lower than 6%, all E% lower than 10% and the averaged E% lower than 5%. Contrarily, the results obtained for the total number of living cells were not encouraging. All the coefficients of variation values were higher than 10% and the average E% was higher than 15%.

*Conclusion*
The results obtained confirmed that TB assay is a very precise method when used to estimate cell viability. On the contrary, for culture population the highly CV and E% values suggested that the method could be inappropriate to estimate the total number of cells despite it is widely used.

# AnaSP: a software suite to automatically analyse spheroid used in high throughput experiments

Piccinini Filippo[1], De Santis Ilaria[2], Angeli Davide[2], Bevilacqua Alessandro[1,3]

[1]Advanced Research Center on Electronic Systems (ARCES), University of Bologna, Bologna, Italy
[2]School of Biology, Department of Biological, Geological and Environmental Sciences, University of Bologna, Italy
[3]School of Engineering and Architecture, Department of Computer Science and Engineering (DISI), University of Bologna, Italy

*Objective*
Today, more and more biological laboratories use 3D cell cultures and tissues grown in vitro, such as multicellular spheroids, as a model of in vivo tumours. However, a lack of methods for quantitative analysis limits the usage of 3D cultures as model for routine experiments. In the last years, several methods have been proposed in literature to perform high-throughput experiments employing spheroids by automatically computing different morphological parameters (e.g., diameter, volume and sphericity), then correlated to proliferation and vitality of the cells composing the 3D aggregates. So far, the cost of the systems required has made the solutions proposed affordable only for a limited subset of laboratories. In this work, we proposed and validated AnaSP, a software suite for automatically computing morphological parameters of spheroids, by simply analysing brightfield images manually acquired with a common widefield microscope.

*Materials and Methods*
To validate the segmentation algorithm implemented, we used 150 images of spheroids of different cell lines, built for different high content screenings by using an antigravity bioreactor and several different hanging drop plates. The ground-truth segmentation was manually obtained exploiting a professional digital graphic tablet.

*Results*
After assessing the computational performance of AnaSP, two sets of experiments were performed to validate the approach proposed. In the first experiment, we computed the absolute error of the different parameters estimated, obtaining values lower than 6%. In the second, we estimated precision and sensitivity of the binary masks automatically obtained by segmenting the different spheroids, achieving values higher than 97%.

*Conclusion*
The results prove that AnaSP can be effectively used to perform spheroid-based high-throughput experiments, besides occasional experiments with limited number of spheroids and standard entry-level instrumentations. AnaSP is distributed as an open source software tool so to encourage even small laboratories to work with 3D cultures.

# Graphene NanoPlatelets (GNPs) in nanomedicine based cancer therapies

Ponticelli Gennaro Salvatore[1,2], Colone Marisa[1], Calcabrini Annarica[1], Rago Ilaria[2], De Bellis Giovanni[2], Sarto Maria Sabrina[2], Stringaro Annarita[1]

[1]Italian National Institute of Health, Department of Technology and Health, Rome, Italy
[2]Research Center on Nanotechnology Applied to Engineering of Sapienza (CNIS), SSNLab, Sapienza University of Rome, Italy

New nanomaterials, especially graphene-based nanomaterials, have had a strong development in the last years in many application fields, and only recently in biomedicine, giving rise to the term nanomedicine. The change in the physicochemical and structural properties of engineered nanomaterials could be responsible for a number of material interactions that could lead to toxicological effects. Thus, several nanomaterials characteristics, such as shape, aggregation, surface coating, and solubility, can culminate in ROS generation, which is currently the best-developed paradigm for nanoparticle toxicity. Herein, we report on the interaction of stable and evenly dispersed exfoliated Graphene NanoPlatelets (GNPs), 1-15 nm thick flakes, constituted of 3-48 layers of graphene, obtained using an ultrasonic bath for different times (30 min, 50 min and 70 min) with human breast adenocarcinoma cells (SKBR3 and MDA-MB-231), for 24-48-72 hrs. Biocompatibility of nanoplatelets has been evaluated by MTT assay while cell membrane damage has been detected using Trypan Blue assay. GNPs particles were more cytotoxic in SKBR3 than MDA-MB-231 cells suggesting a cell phenotype-dependent effect. These results also suggest that ROS generation and the induction of oxidative stress seem to be a possible mechanism of toxic effects, probably due to sharpening of the edges of the nanoplatelets, and possibly by stronger contact interaction with the cell membrane. Furthermore, light microscopy observations and scanning electron microscopy analysis were used to gain understand on the mechanism of cell-nanoplatelets interaction. Our studies carried out with interdisciplinary approaches among chemistry, biology and engineering, can contribute to the mechanistic understanding of graphene-based platforms for bio- and nanomedicine applications.

# Targeting of G-quadruplex telomeric complex by EMICORON has a strong antitumor efficacy against advanced models of human colon cancer

Porru Manuela[1], Artuso Simona[1], Zizza Pasquale[1], Bianco Armandodoriano[2], Franceschin Marco[2], Diodoro Maria Grazia[1], Passeri Daniela[3], Orlandi Augusto[3], Biroccio Annamaria[1], Leonetti Carlo[1]

[1]Regina Elena National Cancer Institute, Experimental Chemotherapy Laboratory, Rome, Italy
[2]University of Rome La Sapienza, Department of Chemistry, Rome, Italy
[3]University of Rome Tor Vergata, Department of Biopathology and Image Diagnostics, Anatomic Pathology Institute, Rome, Italy

New anti-telomere strategies represent important goals for development of selective cancer therapies. In particular, the Gquadruplex (G4) structure, an unusual conformation of the telomeric DNA, plays important roles in some biological events. Recently we have identified a novel G4 ligand, EMICORON. We demonstrated that EMICORON had a high selectively for the G4 structure, efficiently inhibited telomerase, caused telomere damage and inhibition of proliferation selectively in transformed and tumor cells.

*Objective*
Based on these results, our aim was to evaluate the antitumoral effect of this novel G4 ligand on advanced models of human colon cancer.

*Material and methods*
Experiments were performed to identify in vivo the maximum tolerated dose (MTD) of the compound given orally and toxicological profile by immunohistochemical (IHC) analysis. The efficacy of EMICORON was studied on human colon xenografts as orthotopic rectal cancer and in a disseminated neoplasia by intrasplenically injection of bioluminescent HCT116 tumor cells. Moreover, experiments were done on patient-derived xenografts (PDXs) by injection of tumor tissue from patients. Finally, IHC analysis was performed on tumor tissue to identify biological determinants of EMICORON activity.

*Results*
EMICORON was well tolerated in mice as no body weight loss or toxic deaths were observed. Moreover, major organs including bone tissue did not display microscopic difference compared to the mice treated with the vehicle. EMICORON reduced the colonization of HCT116-LUC2 cells at lymphnodes, intestine, stomach and especially liver. Interestingly, EMICORON has a marked antitumor activity on advanced preclinical models such as orthotopic colorectal cancer and PDXs derived from a patient unresponsive to chemotherapy. Finally, the inhibition of angiogenesis and proliferation were key determinant of EMICORON antitumoral activity.

*Conclusion*
Our results identify EMICORON as a new G4 with a promising antitumor activity on relevant experimental models of human colon cancer and warrant for further studies of EMICORON-based combination treatments.

# Chronic myeloid leukaemia-derived exosomes promote tumour growth through an autocrine mechanism

Raimondo Stefania, Saieva Laura, Corrado Chiara, Fontana Simona, Flugy Anna, De Leo Giacomo, Alessandro Riccardo

University of Palermo, Dipartimento di Biopatologia e Biotecnologie Mediche e Forensi, Sezione di Biologia e Genetica, Palermo, Italy

*Objective*

Cancer cells can generate their own signals in order to sustain their growth and survival and recent studies have revealed the role of cancer derived-exosomes in modulating cancer cell behaviour. The aim of the present work is to evaluate the autocrine role of exosomes derived from Chronic Myeloid Leukaemia (CML) cells.

*Materials and Methods*

Cell line used in experiments is LAMA84, a human CML cell line. MTT, Brdu and colony formation assays were performed after up to 1 week of exosomes treatment. NOD-SCID mice were subcutaneously injected with LAMA84 and exosomes. Western Blot and Real Time PCR analysis were performed in in vitro and in vivo samples to assess the expression of pro-and anti-apoptotic molecules, as well as the signal transduction pathways. TGF-β1 receptor inhibitor was used on exosomes-treated cells.

*Results*

CML cells exposed to CML exosomes, show a dose dependent increased proliferation compared with controls. Treatment of mice with exosomes caused a greater increase in tumour size compared with control (PBS-treated mice). Real time PCR and western blot analysis showed an increase of mRNA and protein levels of anti-apoptotic molecules and a reduction of the pro-apoptotic molecules both in in vitro and in vivo samples. Furthermore, we found that TGF- β1 was enriched in CML-exosomes and that exosomes- stimulated proliferation of leukaemia cells, as well as the exosome-mediated activation of the anti-apoptotic phenotype, could be abrogated by blocking TGF-Ð1 signalling.

*Conclusion*

CML derived-exosomes promote, through an autocrine mechanism, the proliferation and survival of tumour cells by activating anti-apoptotic pathways. This mechanism is dependent by a ligand-receptor interaction between TGF-β1, in CML exosomes, and TGF- β1 receptor in CML cells. Our data underline the importance of evaluating the role of leukaemia-derived exosomes for the development of combinatorial therapies that potentiate the effect of Imatinib treatment.

# CXCR4 cyclic peptide antagonist (PepR) – conjugated liposomes (PL–PepR): efficacy and specificity improvement

Caterina Ierano[1], Portella Luigi [1], Lusa Sara[2], D'Alterio Crescenzo[1], Salzano Giuseppina[2], Napolitano Maria[1], Buoncervello Maria[3], Spada Massimo[3], Macchia Daniele[3], Barbieri Antonio[1], Luciano Antonio[1], Gabriele Lucia[3], De Rosa Giuseppe[2], Scala Stefania[1]

[1]Istituto Nazionale per lo Studio e la Cura dei Tumori, Naples, Italy
[2]Facoltà di Farmacia, Naples, Italy
[3]Istituto Superiore di Sanità, Rome, Italy

*Objective*

CXCR4 is overexpressed in multiple tumors regulating metastatic dissemination. A new class of cyclic peptides antagonist for CXCR4 receptors was recently developed. To improve peptide efficacy and increase its delivery to target cancer cells the most active antagonist, Pep R, was coupled to PEGylated liposomes (PL).

*Materials and Methods*

PepR conjugated to the liposomes (PL-PepR) were prepared starting by athiolated derivative of antiCXCR4 peptides coupled to the pre-formed PL. Doxorubicin (DOX) was then encapsulated by remote loading method. PL-PepR was evaluated through migration assay in A498 human renal cancer cell line in vitro and in an experimental animal model of pulmonary metastasis development in vivo. DOX-encapsulating PL-PepR was evaluated in CXCR4 positive cells A498 and HT29 (colon cancer cell line) versus negative CXCR4 expressing cells FB-1(human anaplastic thyroid cell line), as mean cellular fluorescence. The cytotoxic effect of the PL-DOX-PepR was examined in A498 and in HT29 cells. Finally, Lipo-DOX-PepR was evaluated in vivo polmonary metastasis formations in C57/BL mice injected with B16-CXCR4 cells.

*Results*

In vitro studies, PL-Peptide R inhibited migration CXCL12-induced in A498 human renal cancer cells-CXCR4 expressing more efficiently than Peptide R alone.To validate the PL-PepR efficacy in vivo, metastases development assays were conducted. A significant reduction in lung metastases was detected in mice treated with PL-PepR even with lower dose of the PL-PepR (0.1mg/kg) compared to the usually used (2mg/kg). Moreover, a CXCR4 dependent higher DOX accumulation was registered in CXCR4 positive cells, A498 and HT29 resulting in a specific higher cytotoxicity. This was confirmed in vivo experiments, in which PL-DOX-PepR reduced lung metastases compared to PL-Doxo treated mice.

*Conclusions*

Liposomes conjugated-rationally designed CXCR4 antagonist were more efficient in inhibiting CXCR4 in vitro and in vivo. Moreover, PL-PepR loaded with a chemotherapeutic drug, such as DOX, demonstrated an enhanced drug accumulation to CXCR4 expressing tumor.

# Radioresistance of breast cancer T47D cells grown as 3D cultures

Sega Michela, Chignola Roberto

University of Verona, Department of Biotechnology, Verona, Italy

The clonogenic assay is still considered the method of choice to determine the gross effects of radiation therapy on tumour cells. However, this approach has several limitations among which the fact that it does not allow to measure the dynamics of cell recovery/death after treatment, dynamics that are heavily influenced by the tumour microenvironment.

*Objective*
To investigate the dynamic effects of radiations on the relative radioresistant T47D cells grown as multicellular spheroids.

*Materials and Methods*
Cells from the human breast cancer cell line T47D were grown as multicellular tumour spheroids using the liquid overlay technique. Spheroids of about 400 μm diameter were exposed to gamma radiation (2-12 Gy), then plated individually in 6-well plates for regrowth experiments. Image analysis was performed using the ImageJ software. Cell morphology was analysed by Giemsa stain and cell viability was measured by Trypan blue and ATP assays.

*Results*
T47D spheroids showed unexpected re-growth kinetics even after the administration of radiation doses > 6 Gy. Growth delays could last more than 600 hours post radiation exposure. Viability and morphological analyses showed that cells in the regrowth area around the spheroid could stop cycling for several weeks while remaining alive. Pluri-nucleated giant cells arose in the population after therapy and progressively disappeared. Giant cells were no more visible at the time when cells started again to proliferate. Overall, cell survival did not decrease with radiation dose as expected by the classical Linear Quadratic model.

*Conclusion*
T47D spheroids are radioresistant and can be used as an interesting cell model to study non-conventional response patterns to radiotherapy. Our preliminary results also indicate that the effect of the radiation can be underestimated by classical clonogenic assay.

# Triggering of CD99 by 0662 mAb induced endocytosis of Ras and cell death in Ewing sarcoma

Terracciano Mario[1], Manara Maria Cristina[1], Morrione Andrea[2], Scotlandi Katia[1]

[1]Experimental Oncology Laboratory, Istituto Ortopedico Rizzoli, Bologna, Italy
[2]Department of Urology and Biology of Prostate Cancer Program, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, USA

*Objective*
CD99 is a cell surface molecule with diagnostic and therapeutic implications in Ewing sarcoma (EWS). Engagement of CD99 with monoclonal antibodies (mAbs) leads to a massive death of EWS cells. MDM2 degradation and p53 reactivation are part of the mechanisms but a complete picture of intracellular trafficking is still far from being defined. In this study, we determined the CD99 fate together with modulations in relevant intracellular signaling pathways.

*Materials and Methods*
ELISA, western blotting and nichel beads pull-down assays were used to study CD99 stability. Immunofluorescence and confocal microscopy were used to establish internalization and intracellular location of CD99 after engagement with mAbs. Protein-protein interactions were established by immunoprecipitation (IP) assays.

*Results*
Imaging clearly defined how CD99 first colocalized either with clathrin and caveolin-1, both proteins actively involved in the early phases of endocytosis process, then with Lamp-1, a lysosome marker present of the membrane of these organelles, resulting also ubiquitinated during the process. Since the induction of MAPK family members by the engagement of CD99 has been documented before, we investigated the modulation of Ras GTPase upon mAb treatments: we found out that, after its initial upregulation, Ras resulted binded to CD99 and shared also the same destiny, undergoing a lysosome-mediated degradation.

*Conclusions*
The whole death mechanism began with CD99 internalization, which was mainly processed into the lysosomes. Cells tried to counteract the death stimulus by upregulating Ras, then CD99 dragged it into the internalization process leading the small GTPase to a lysosome-mediated degradation.

# Micelle-loaded mesenchymal stromal cells: a new carrier-in-carrier device for curcumin delivery and targeting

Tripodo Giuseppe[1], Perteghella Sara[1], Vigani Barbara[1], Mandracchia Delia[2], Trapani Adriana[2], Tosca Marta Cecilia[3], Chlapanidas Theodora[1]

[1]University of Pavia, Department of Drug Sciences, Pavia, Italy
[2]University of Bari, Department of Pharmacy, Pavia, Italy
[3]Niguarda Hospital, Struttura Semplice Tissue Therapy, Milan, Italy

In this work, mesenchymal stromal cells (MSCs) together with curcumin loaded INVITE MC micelles, formed a carried-in-carrier system which could be identified as a double drug delivery system. The first order drug carrier is represented by the INVITE MC micelle that efficiently incorporated the highly hydrophobic drug curcumin, while the second order carrier is represented by the MSCs that could also assure an adequate drug targeting due to their innate ability to reach the injured tissues. The uptake of the INVITE MC micelles by MSCs resulted effective and quick: the uptake, independently from the tested concentrations, was detected at just 30 minutes of incubation, and no time-dependence was evidenced. The localization of the INVITE MC inside the MSCs was cytosolic as demonstrated by confocal microscopy differently from "naked" curcumin which was found also in the cell nucleus. Furthermore, "naked" curcumin resulted highly cytotoxic for the MSCs, while curcumin-loaded INVITE MC resulted highly cytocompatible. Finally, MSCs loaded with micelles are able to release the entrapped drug.
Appling this integrated biological-technological drug delivery approach, the loaded MSCs could be directly administered as an Advanced Therapy Medicinal Product, or to obtain biological drugs such as microvesicles, exosomes, or conditioned medium. The INVITE MC/MSCs carrier-in-carrier system could become a new paradigm in drug delivery.

# Role of HIF-1α, HJURP, TG2 to predict the evolution of In Situ Breast Cancers

Tumedei Maria Maddalena[1], Scarpi Emanuela[2], Zoli Wainer[1], Rengucci Claudia[1], Serra Luigi[3], Curcio Annalisa[4], Buggi Federico[4], Folli Secondo[4], Rocca Andrea[5], Maltoni Roberta[5], Puccetti Maurizio[6], Amadori Dino[5], Silvestrini Rosella[1], Bravaccini Sara[1]

[1]Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy
[2]Unit of Biostatistics and Clinical Trials, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy
[3]Pathology Unit, Morgagni-Pierantoni Hospital, Forlì, Italy
[4]Breast Surgery Unit, Morgagni-Pierantoni Hospital, Forlì, Italy
[5]Department of Medical Oncology, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Meldola, Italy,
[6]Pathology Unit, Santa Maria delle Croci Hospital, Ravenna, Italy

*Objective*
There is evidence to suggest that DCIS (ductal carcinoma in situ) is a heterogeneous group of lesions with distinct differences in terms of morphology, biology, and evolutive behaviour. The current use of mammographic screening has led to earlier diagnosis which has increased the number of small IC (invasive cancer) or DCIS tumors found. Traditional pathological factors are not capable of identifying which in situ lesions will recur and/or progress to invasive carcinoma. The aim of the present study was to investigate the clinical significance of conventional and new markers in in situ breast cancer and in the tumor microenvironment, and to assess any correlation with clinical outcome in patients homogeneously treated with surgery and post-surgery radiation therapy.

*Matherials and Methods*
We investigated 3 biomarkers by immunohistochemistry in tumor cells and surrounding stroma of 44 patients with CIS: TG2 (transglutaminase 2), HJURP (Holliday junction recognition protein), and HIF-1α (hypoxia inducible factor-1 alpha).

*Results*
TG2 was more highly expressed than the other two markers and significantly more so in stromal than in tumor cells. HIF-1α evaluation showed a higher expression in both tumor and stromal cells in patients with relapsed G3 tumors, indicating a potential role of this marker in CIS evolution. A greater than sevenfold higher risk of relapse (P = 0.050) was observed in patients highly expressing HJURP in stroma and a tenfold higher recurrence risk (P = 0.026) was seen in those with a higher stromal HIF-1α expression. An important increase in risk accuracy (AUC 0.80) was obtained when HIF-1α and HJURP were evaluated together.

*Conclusion*
Despite the limited number of relapsed patients, we formulated some hypotheses on the factors responsible for malignant evolution and recurrence which are now being tested in a large case series with a longer follow-up.

# RNA interference against PCMT: new strategy to improve pharmacological treatment of glioblastoma

Vigorito Carmela, Capasso Rosanna, Luce Amalia, Zappavigna Silvia, Gigantino Vincenzo, Ingrosso Diego, Caraglia Michele

Department of di Biochemistry, Biophisics and General Pathology, Second University of Naples, Naples, Italy

*Introduction*

Glioblastoma (GBM) is the most common brain cancer in adults, characterized by rapid progression and poor prognosis, and the least responsive to therapy. Over the last 10 years, only two chemotherapeutic agents, carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU implant) and temozolomide (TMZ), an imidazotetrazine derivative of dacarbazine, have received regulatory approval for treating malignant gliomas. PCMT1(EC 2.1.1.77) is a SAM-dependent methyltransferase which repairs antiapoptotic proteins, including BclxL, and increases susceptibility to apoptosis in hepatocarcinoma cells.
Zoledronic acid (ZOL) are a potent amino-bisphosphonate used for the treatment of bone metastases with recently reported antitumor activity.
AIM: We analyzed PCMT1 expression levels and its molecular targeting as a means to increase susceptibility of GBM to apoptosis, combined with treatment with chemotherapeutic agents.

*Methods*

PCMT1 compartimentalization and expression levels were analyzed in situ in slides from GBM specimens by immunohistochemistry. Sensitivity to apoptosis and to various treatments were studied using various GBM cell lines derived from human GBM. Targeting of PCMT1 was accomplished in vitro on GBM cell lines by using anti PCMT1 siRNA against 3' and 5' or ORF carried by Hyperfect Transfection reagent. (Qiagen).

*Results*

PCMT1 expression and subcellular distribution in various GBM cell lines reflects grade and malignacy of originating tumors. We found that PCMT-silenced GBM cell lines, by using anti-PCMT siRNA, showed enhanced sensitivity to apoptosis induced by several drugs, including doxorubicin and themozolomide. Treatment of PCMT-silenced GBM cell lines with free ZOL and encapsulated ZOL showed increased susceptibility to apoptsis.

*Conclusions and Perspectives*

In the near future we are planning to use miRNA 15a and 16-1 mimics to improve the effects of gene therapy based on the pleiotropic effects of these two miRNAs against PCMT1 and Bcl2. Studies on the effects of combined anti-PCMT1/chemotherapy on mouse xenograft models carrying human GBM are also planned.

# Canine osteosarcoma cancer-stem like cells: a comparative study model in oncology

Vito Guendalina[1], Campanella Chiara[1], Ratto Alessandra[1], Razzuoli Elisabetta[1], Florio Tullio[2], Monica Gatti[2], Federica Barbieri[2], Lorella Maniscalco[3], Raffaella De Maria[3], Paolo Buracco[3], Angelo Ferrari[1]

[1]Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Italy
National Reference Center for Veterinary and Comparative Oncology (CEROVEC), Genoa, Italy
[2]Section of Pharmacology, Department of Internal Medicine, University of Genoa, Italy
[3]Department of Veterinary Sciences, University of Turin, Italy

*Objective*
Emerging evidence suggests that cancer stem cells (CSCs), a small cell subpopulation within tumors, are responsible for cancer growth, drug-resistance, recurrence and metastasization, therefore representing candidate therapeutic targets. The objective of this study was to characterize CSCs isolated from canine osteosarcoma (OSA) cell lines, and investigate their responsiveness to metformin, a diabetes drug undergoing investigation as a potential treatment for different human cancers. OSA is the most common solid bone cancer type both in dogs and humans, sharing similar histopathological, clinical and biological features. Thus OSA-CSC may be a suitable model for oncological research.

*Materials and Methods*
OSA cells, derived from canine osteoblastic productive and chondroblastic tumors, were enriched in CSCs by culturing them a serume-free permissive medium (supplemented with EGF and bFGF). Self-renewal and stem marker (CD133, CXCR4, Oct4) expression were evaluated by sarcosphere forming-assay, and immunofluorescence, respectively; cell growth and sensitivity to metformin was tested by MTT assay and the in vivo tumorigenicity by s.c. injection of cells into NOD/SCID mice.

*Results*
OSA-CSC cultures homogeneously expressed stem markers, showed slow proliferation rate, generating non-adherent colonies, a self-renewal index. When shifted to serum-containing medium, sarcospheres gave rise to adherent monolayers. Notably, only cells maintained in stem-permissive conditions were highly tumorigenic, recapitulating the canine OSA characteristics in mice. Conversely, the corresponding culture exhibited a significantly lower tumor-formation ability. In vitro proliferation rate and in vivo tumor growth were consistent with the aggressiveness of canine tumor of origin, higher in chondroblastic than in osteoblastic OSA. Moreover, metformin induced a significant anti-proliferative effect on OSA CSCs.

*Conclusion*
Our preliminary results confirm the presence of tumorigenic stem cells, sensitive to metformin, in canine OSA and suggest the reliability of isolation and enrichment of CSCs from OSA cell cultures as an innovative model to search for novel CSC-targeting drugs and highlights the role of canine tumors in COMPARATIVE oncology.

# SPERA – Sperimentare per curare: reasons for research

De Girolamo Paolo[1], Piscitelli Marta[2], Gentili Elena[3]

[1]Università degli Studi "Federico II", Napoli, Italy
[2]Enea, Roma, Italy
[3]SPERA – Sperimentare per curare, Roma, Italy

In Italy, scientific research is often perceived as a theoretical activity with no application to everyday life - a reason for this is the lack of a clear and correct scientific divulgation. The public opinion seems to be particularly hostile to animal sperimentation, the aspect of biomedical research that is most frequently misjudged and misspoken of. This also depends on the strong anti-animal sperimentation campaigns conducted by animalistic associations that play on the public's feelings through sensational and often inaccurate news. This whole situation can seriously damage research itself and its applications.

That is why in October 2013 AISAL, the Italian Association for Laboratory Animal Science, launched the project "SPERA – Sperimentare per curare", which aims at providing accurate and understandable information, addressing all citizens, both experts and non-experts, adults as well as students, politicians as well as voters.

For the first time, a quite large group of researchers with different experiences in different fields has assembled to work together at this project. Believing in networking among scientific associations, patients associations and schools, SPERA intends to grow the numbers of its participants to eventually evolve into a Scientific Federation.

SPERA represents those who are morally and scientifically committed to the proper use of laboratory animals in scientific research and it supports their role through a campaign to raise awareness about the aims and instruments of scientific research and the results it has accomplished so far. Therefore, SPERA intends to promote curiosity with a series of different projects that would involve scientists and students of all levels.

SPERA's main goal is to give people, the ultimate beneficiary of biomedical research, the tools necessary to develop a personal critic opinion on the subject of animal sperimentation, by encouraging an open dialogue and debate that won't resort to sensationalisms and prejudices.

# Mesenchymal stromal cells primed with paclitaxel as a potential therapy for the treatment of metastatic melanoma

Artuso Simona[1], Leonetti Carlo[1], Orlandi Augusto[2], Passeri Daniela[2], Ceserani Valentina[3], Alessandri Giulio[3], Benetti Anna[4], Dessy Enrico[4], Berenzi Angiola[4], Pascucci Luisa[5], Coccè Valentina[6], Bonomi Arianna[6], Sisto Francesca[6], Pessina Augusto[6]

[1]Preclinical Experimental Laboratory, Regina Elena National Cancer Institute, Rome, Italy
[2]Department of Biopathology and Image Diagnostics, Anatomic Pathology Institute, University of Rome "Tor Vergata", Italy
[3]Cellular Neurobiology Laboratory Department of Cerebrovascular Disease IRCCS Neurological Institute Carlo Besta, Milan, Italy
[4]Deparment of Pathological Anatomy, Ospedali Civili di Brescia and University of Brescia, Italy
[5]Department of Veterinary Medicine, University of Perugia, Italy
[6]Department of Biomedical, Surgery and Dental Sciences, University of Milan, Italy

Our previously observation that mesenchymal stromal cells (MSCs) have the ability to uptake Paclitaxel (PTX), release the drug and produce cytotoxic effects in vitro against melanoma cells, led us to investigate the potential antitumor efficacy of this strategy in vivo. To this purpose, we evaluated if the systemic administration of PTX loaded by MSCs (SR4987) could reduce the metastatic dissemination of the B16 murine melanoma.

Mice were injected i.v. with melanoma cells and treated i.v. (days 5, 10, 15 after tumor cells injection) with saline (controls), PTX, SR4987 and with SR4987 primed with PTX. Immunohistochemical (IHC) analysis on formalin-fixed lungs was performed to identify the presence of MSCs by using the monoclonal anti-Sca-1/Ly6A/E antibody.

The results showed that treatment with SR4987/PTX was highly effective in reducing the metastatic ability of B16 melanoma. In fact, SR4987/PTX administration decreased of 90% the number of metastasis compared to the about 70% reduction observed after the treatment with PTX alone. The number of lung nodules after SR4987/PTX treatment was significantly different ($P<0.001$) compared to PTX, SR4987 alone or untreated group.

IHC analysis performed on lung sections demonstrated the antimetastatic effect of SR4987/PTX could be related to the ability of MSCs to home in tumor microenviroment and release the drug. In fact, we observed a significant increase of Sca-1+ cells in SR4987 and SR4987/PTX treated mice lungs compared to untreated and PTX-treated groups. Interestingly, in mice treated with SR4987 a significant presence of Sca-1+ cells was observed nearby vessels and at periphery of metastatic nodule and notably, after treatment with SR4987/PTX, Sca-1+ cells were detected also in the lumen of microvessels infiltrating metastatic nodules.

In conclusion, our results suggest that PTX delivered by MCSs could be a promising strategy for the treatment of melanoma that presently is a target of poor pharmacological weapons in humans.

# Alteration of gene expression induced by Tributyltin in porcine Aortic Vascular Precursor Cells

Bernardini Chiara, Zannoni Augusta, Bertocchi Martina, Forni Monica

University of Bologna, Department of Veterinary Medical Sciences – DIMEVET Bologna, Italy

*Objective*

Endocrine disruptor chemicals (EDCs) such as Tributyltin (TBT) are toxic compounds widely present in the environment with harmful effects including perturbation of transmembrane ion gradients, oxidative stress and apoptosis. TBT may constitute a risk factor for cardiovascular disease inducing morphological vascular alteration and impairing coronary vascular reactivity. Recently we described the isolation of porcine Aortic Vascular Precursors Cells (pAVPCs), these cells have the phenotypic characteristics of mesenchymal cells, and functional properties of pericytes. Few studies have investigated the TBT effect on stem cells and none has been done on vascular precursor cells. Aim of the present study was to evaluate the influence of TBT on pAVPCs gene expression.

*Materials And Methods*

pAVPCs were seeded in a 24 wells (3x105/well) plate and exposed to increasing doses of TBT (10, 100, 500 nM) for 48h, cytotoxicity was evaluated and gene expression of mesenchymal (CD90, CD73, CD105), perycite (NG2, Nestin, PDGFÐR, alpha-SMA) angiogenetic (VEGF, Flt-1) and stem cell (NANOG) markers were investigated by qPCR analysis.

*Results*

TBT reduced cell viability reaching a mortality rate of approximately 30% at the highest dose, furthermore TBT influenced the expression of most of the studied genes. Mesenchymal markers increased at the lowest dose (10 nM) and decrease at the highest dose (500nM), otherwise the expression of NG2, Nestin and PDGFÐR decreased at all three doses studied. The most sensitive gene was alpha-SMA, with an increase of approximately 7 times at the lowest dose and a strong reduction at the higher dose. Angiogenetic and stem cell markers did not seem affected.

*Conclusions*

Overall TBT perturbed the expression of pAVPCs' genes pattern, further investigation will be necessary to clarify the structural and functional effects of these alterations and the impact of these changes in relation to differentiation potential.

# Endothelial differentiation of porcine Aortic Vascular Precursor Cells

Bertocchi Martina[1], Zaniboni Andrea[1], Bernardini Chiara[1], Zannoni Augusta[1], Alessandri Marco[3], Calzà Laura[2,3], Bacci Maria Laura[1], Forni Monica[1]

[1]University of Bologna, Department of Veterinary Medical Sciences - DIMEVET Bologna, Italy
[2]University of Bologna, Department of Pharmacy and Biotechnology - FaBiT Bologna, Italy
[3]Health Sciences and Technologies - Interdepartmental Center for Industrial Research, University of Bologna, Ozzano Emilia, Bologna, Italy

*Objective*

Vascular Stem Cells (VSCs) are recently defined as cells reside within the vessel wall, which show the typical mesenchymal characteristics and can differentiate into smooth muscle cells (SMCs) and endothelial cells (ECs), the cell types that constitute a functional blood vessel. Recently, we described the isolation of vascular precursor cells from the tunica media of porcine thoracic aortic (porcine Aortic Vascular Precursor cells, pAVPCs). These cells have mesenchymal phenotypic characteristics, pericytes typical functional properties and are able to differentiate spontaneously into smooth muscle cells. The aim of this study was to assess the differentiation potential of pAVPCs toward the endothelial lineage.

*Materials and Methods*

pAVPCs were seeded in a 24 well plate (5000 cells/cm2) and cultured in Endothelial Differentiation Medium (EDM) (human Endothelial Serum Free Medium supplemented with 5% FBS and 50 ng/mL of human recombinant Vascular Endothelial Growth Factor [hVEGF]) or in Perycite Growth Medium (PGM) as control. After 21 days of culture, the expression level of differentiated endothelial cells markers, CD31, VE-Cadherin, von Willebrand Factor (vWF) and endothelial Nitric Oxide Synthase (eNOS) was analyzed by qPCR. Expression of CD31 was also tested by immunofluorescence.

*Results*

The gene expression analysis revealed a significant increase of expression levels of all the endothelial markers in cells cultured in EDM respect to the control (PGM), with VE-Cadherin and CD31 showing an increase of 33 and 22 times, respectively. Immunofluorescence analysis of CD31 revealed that only cells cultured in EDM expressed the protein with the classical membrane localization.

*Conclusions*

We demonstrated that pAVPCs, grown in specific endothelial medium and stimulated with VEGF, are able to differentiate toward the endothelial cells phenotype. Therefore, according to the recent definition of VSC, the pAVPCs could be considered a population of VSC-like cells.

# Nanovesicles from mesenchymal stem cells: experimental assessment of an innovative therapeutic approach for ALS

Bonafede Roberta, Scambi Ilaria, Farinazzo Alessia, Turano Ermanna, Bonetti Bruno, Mariotti Raffaella

University of Verona, Department of Neurological and Movement Sciences, Verona, Italy

*Objective*

Therapeutic strategies for the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS) are actually minimally effective on patients' survival and quality of life. Stem cells are becoming increasingly important in the treatment of neurodegenerative diseases and their beneficial effect seem to be due to a paracrine effect via the release of nanovesicles (NV). Here we want to assess the neuroprotective effect of NV derived from mesenchymal adipose stem cells (ASC; ASC-NV) on motoneuronal cell culture.

*Materials and Methods*

Murine ASC were isolated from inguinal adipose tissues of C57BL/6 mice; NV were obtained from ASC supernatants after filtration and ultracentrifugation and were identified by electron microscopy and immunoblotting. Neuroblastoma cell line (SH-SY5Y), primary cultures of hippocampal neurons and motoneuronal cell line (NSC-34) were used for identification of the best H2O2 and ASC-NV concentration. Naïve NSC-34 and NSC-34 cells transiently transfected with mutant SOD1(G93A) human gene were used for evaluation of neuroprotective effect of ASC-NV. Cell death were evaluated with DAPI and anti-caspase3 antibody.

*Results*

To evaluate the neuroprotective effect of ASC-NV in vitro, we first set up the protocol of reproducible isolation of ASC-NV, identified the optimal dose of ASC-NV which protect neural cells from apoptosis, the cell-plated density and the optimal concentration of H2O2 used as pathological insult. These parameters was assessed on SH-SY5Y, primary culture of hippocampal neurons and on NSC-34. Interestingly, on naïve NSC-34 cells and on NSC-34 cells transiently transfected with human mutant SOD1(G93A) gene, the administration of ASC-NV in the culture medium protected cells from oxidative damage (H2O2), with a 30% increase of cell viability.

*Conclusion*

Our results on NSC-34 motoneuronal cells line, naive or transiently transfected, point out the neuroprotective role of ASC-NV from oxidative damage. ASC-NV could be a substitute for cell-based therapy and represents a promising approach in neurodegenerative disorders.

# Human amniotic mesenchymal stromal cells (hAMSCs) as potential vehicle for drug delivery in cancer therapy: an in vitro study

Bonomi Arianna[1], Parolini Ornella[2], Silini Antonietta[2], Coccè Valentina[1], Vertua Elsa[2], Romele Pietro[2], Cavicchini Loredana[1], Sisto Francesca[1], Alessandri Giulio[3], Pessina Augusto[1]

[1]Department of Biomedical, Surgical and Dental Sciences, Università degli Studi, Milan, Italy
[2]Centro di Ricerca E. Menni Fondazione Poliambulanza-Istituto Ospedaliero, Brescia, Italy
[3]Cellular Neurobiology Laboratory, Department of Cerebrovascular Diseases, Fondazione IRCCS Neurological Institute C. Besta, Milan, Italy

*Objective*
We've previously shown that mesenchymal stromal cells (MSCs) from bone marrow (BM) can uptake the anti-cancer and anti-angiogenic drug Paclitaxel (PTX) and then release it to affect tumor proliferation both in vitro and in vivo. As BM-MSCs show limitations (invasive collection procedure, reduced recovery with donor age) we investigated alternative MSCs sources, such as amnion. In this in vitro study, we evaluated if mesenchymal stromal cells derived from human amniotic membrane (hAMSCs) showed the same ability of BM-MSCs to uptake/release PTX and to inhibit tumor proliferation.

*Materials And Methods*
hAMSCs, were obtained by collagenase treatment of the amniotic membrane of four term human placenta and were characterized for CD expression and differentiation ability towards classic mesodermal lineages. Sub-confluent cultures of hAMSCs were treated with 2 µg/ml of PTX, previously shown to inhibit cell proliferation without affecting cell viability. hAMSCs were recovered after 24h according to a standard operating procedure, sub-cultured and their conditioned media (hAMSCsPTX-CM) collected at different time points. hAMSCsPTX-CM were tested for their anti-proliferative activity on two human tumor cell (TCs) lines (adenocarcinoma CFPAC-1 and acute lymphoblastic leukemia MOLT-4) selected in our laboratory as reference TCs for their PTX sensitivity.

*Results*
hAMSCs, as previously demonstrated with stromal cells from BM, adipose tissue and dermis, show the ability to uptake and release PTX in culture medium in a time-dependent manner. Indeed, if TCs are cultured in the presence of serial dilutions of hAMSCsPTX-CM, their proliferation is inhibited in a dose-dependent manner.

*Conclusion*
For the first time we show that hAMSCs uptake and subsequently release PTX without any genetic manipulation, thus becoming a potential drug vehicle to be used in cell therapy. Furthermore, because amnion is an easily accessible and high-yielding source without ethical objection, further studies are ongoing to investigate the potential of drug loaded hAMSCs as new tool against cancer and other pathologies.

# Human skin derived fibroblasts as vehicle of anti-tumor agents work well in hypoxic conditions

Coccè Valentina[1], Vitale Alessandro[2], Colombo Silvia[2], Bonomi Arianna[1], Ciusani Emilio[3], Cavicchini Loredana[1], Sisto Francesca[1], Alessandri Giulio[4], Parati Eugenio[4], Brambilla Paolo[5], Brambilla Maura[5], La Porta Caterina A.M.[2], Pessina Augusto[1]

[1]Department of Biomedical, Surgical and Dental Sciences, University of Milan, Italy
[2]Department Bioscience, Molecular Oncology Laboratory, University of Milan, Italy
[3]Department of Diagnostics and Applied Technology, Fondazione IRCCS Neurological Institute Carlo Besta, Milan, Italy
[4]Department of Cerebrovascular Diseases, Fondazione IRCCS Neurological Institute Carlo Besta, Milan, Italy
[5]Department of Diagnostic Services, Desio and Vimercate's Hospital, Italy

*Objective*

The main problem of cancer chemotherapy is to localize it's cytotoxic effect to reduce the collateral toxicity of the drug to the lowest level. An interesting approach is based on the ability of human mesenchymal stromal cells (hMSCs) to migrate into inflammation/injury sites and into tumor stroma. The idea, inspired to the image of the "trojan horse", is to use hMSCs as vehicle to deliver anti-tumor agents. We investigate the ability of human skin derived fibroblasts (hSDFs) to deliver Paclitaxel (PTX) and to inhibit in vitro a human melanoma. Furthermore we verified if PTX uptake-release by hSDFs is modified by hypoxic conditions that often characterize the tumor mass environment.

*Materials And Methods*

hSDFs were treated with PTX according to a standardized procedure by evaluating uptake/release of PTX in normoxic and hypoxic conditions. Cell cycle, apoptosis of hSDFs primed with PTX (hSDFs-PTX) were quantified by cytofluorimetric analyses. Drug release was confirmed by mass spectrometry analysis. Human melanoma cell line (IgR39) was used to quantify the antitumor effect of PTX released.

*Results*

hSDFs are resistant to the cytotoxic effect of PTX so these cells can be primed with high amounts of drug. In vitro antitumor activity of PTX released by hSDFs-PTX was confirmed by transwell and rosette assay and also evaluated by a crystal violet colony growth assay. hSDFs-PTX undergo apoptosis more than untreated hSDFs and hypoxia doesn't induce significant changes of cell cycle pattern and of PTX release.

*Conclusion*

Our study shows that hSDFs incorporate and release PTX with unaffected pharmacological activity against human melanoma growth in vitro. Hypoxia doesn't affect the release of PTX by the programmed death of PTX primed hSDFs . Although in vivo experiments are necessary to confirm these in vitro data, hSDFs could be considered as a potential tool for drug delivery in clinical trial.

# Mesenchymal stromal cells uptake Paclitaxel and release it through membrane vesicles that inhibit in vitro tumor growth

Coccè Valentina[1], Pascucci Luisa[2], Bonomi Arianna[1], Ami Diletta[3], Ceccarelli Piero[2], Ciusani Emilio[4], Viganò Lucia[5], Locatelli Alberta[5], Sisto Francesca[2], Doglia Silvia Maria[6], Parati Eugenio[7], Bernardo Maria Ester[8], Muraca Maurizio[9], Alessandri Giulio[7], Bondiolotti Gianpietro[10], Pessina Augusto[1]

[1]Department of Biomedical, Surgical and Dental Sciences, University of Milan, Italy
[2]Department of Veterinary Medicine, University of Perugia, Italy
[3]Department of Biotechnology and Biosciences, University of Milan Bicocca, Italy
[4]IRCCS Neurological Institute Carlo Besta, Laboratory of Clinical Pathology and Neurogenetic Medicine, Milan, Italy
[5]Ospedale S. Raffaele Scientific Institute, Department of Medical Oncology, Milan, Italy
[6]Department of Physics, University of Milan Bicocca, Italy
[7]IRCCS Neurological Institute C. Besta, Department of Cerebrovascular Diseases, Cellular Neurobiology Laboratory, Milan, Italy
[8]Department of Onco-Hematology,Children's Hospital Bambino Gesù, Rome, Italy
[9]Laboratory Medicine, Children's Hospital Bambino Gesù, Rome, Italy
[10]Department of Medical Biotechnology and Translational Medicine, University of Milan, Italy

*Objective*

In recent studies, we demonstrated that Mesenchymal Stromal Cells (MSCs), cultured in presence of the anticancer drug Paclitaxel (PTX), are able to uptake substantial amounts of the drug without showing significant signs of toxicity, and acquire the capacity to kill tumor cells of different types, located in their proximity, both in vitro and in vivo. Since MSCs constitutively secrete high amounts of membrane vesicles (MVs), we here investigated their role in PTX releasing mechanism.

*Materials and Methods*

The murine SR4987 line was used as MSC model. The release of PTX from SR4987 in the conditioned medium (CM) was checked by HPLC and the anti-tumor activity of both CM and MVs was tested on the human pancreatic cell line CFPAC-1. It is known, in fact, that pancreatic cancers are usually very malignant and insensitive to chemotherapy. MVs were isolated by ultracentrifugation, analyzed by transmission (TEM) and scanning electron microscopy (SEM), and the presence of PTX was evaluated by the Fourier transformed infrared (FTIR) microspectroscopy.

*Results*

SR4987 loaded with PTX (SR4987PTX) secreted a significant amount of PTX and their CM possessed strong anti-proliferative activity on CFPAC-1. At TEM and SEM, SR4987PTX showed an increased number of "vacuole-like" structures and shed a relevant number of MVs, but did not show ultrastructural alterations when compared to untreated SR4987. SR4987PTX-derived-MVs (SR4987PTX-MVs) demonstrated a strong anti-proliferative activity on CFPAC-1. FTIR analysis of SR4987PTX-MVs showed the presence of an absorption spectrum in the corresponding regions of the PTX marker, absent in MVs from control SR4987.

*Conclusion*

Our work is the first demonstration that MSCs are able to package active drugs inside MVs and to specifically transport them to tumor cells. This suggests the possibility to explore a new MV-based approach to drug delivery.

# Role of WNT/beta-catenin up-regulation, cadherins alterations and p-runx2 expression in fibro-osseous lesions of the jaws. A tissue microarray study

Di Domenico Marina[1], Pannone Giuseppe[2], Santoro Angela[2,3], Feola Antonia[1], Iorio Rosamaria[1], Auletta Adelaide[1], Di Carlo Angelina[4], Bucci Eduardo[5], Bucci Paolo [5]

[1]Department of Biochemistry, Biophysic and General Pathology, Second University of Naples, Italy
[2]Department of Clinical and Experimental Medicine, Institute of Pathological Anatomy Section of Oral Pathology, University of Foggia, Italy
[3]Department of Laboratory and Services, Institute of Histopathology and Diagnostic Cytopathology, Fondazione di Ricerca e Cura 'Giovanni Paolo II'-UCSC, Campobasso, Italy
[4]Department of Medico-Surgical Sciences and Biotechnologies "Sapienza" University of Rome, Italy
[5]Department of Neuroscience, Reproductive and Odontostomatological Sciences, Institute of Oral Pathology University of Napoli 'Federico II', Naples, Italy

*Objective*
Among benign fibro-osseous lesions of bone two disease have been well characterized, Fibrous Dysplasia (FD), caused by GNAS mutation and Hyperparathyroidism-Jaw tumor syndrome (HPT-JT), caused by HRPT2/CDC73 mutation. Despite the causal mutations have been already demonstrated, the intracellular mechanism that leads to excessive deposition of fibrous tissue, and alteration of differentiation processes leading to osteomalacia has not yet been fully clarified.

*Matherial and Methods*
Tissue Microarray (TMA) based immunohistochemical expression of β-catenin, CK-AE1/AE3, Ki-67, cadherins (E-cadherin, N-cadherin, P-cadherin, OB-cadherin) and P-RUNX2 has been analysed in archival samples from 9 patients, respectively affected by FD (n.8) and HPT-JT (n.1) and in 7 controls, represented by reactive fibrous tissues and normal remodelling bone.

*Results*
Beta-catenin was strongly up-regulated in FD showing hyper-cellulated pattern, while it was faintly expressed in bone tumors associated to HPT-JT. Furthermore, we showed that loss of expression of OB-Cadherin in osteoblast lineage in FD was accompanied by up-regulation of N-cadherin and P-cadherin, while E-cadherin showed a minor role in these pathological processes. Beta-catenin plays a central role in fibrous tissue proliferation and accompanies the lack of differentiation of osteoblasts precursors in mature osteoblasts in FD.

*Conclusions*
This study on the one hand confirms the complete absence of E-cadherin and OB-cadherin but on the other hand shows that the OB-cadherin loss is vicariate by the considerable increase of N-cadherin expression and by the expression of P-cadherin. P-runx-2 showed over-expression in 6 out 8 cases of FD, both in fibroblastic stroma and in retracted osteoblast and stained moderately positive in the rimming lining osteoblasts in HPT-JT syndrome.

# Mesenchymal Stem Cells from vertebral body and iliac crest bone marrow: Comparison of two tissues and two harvesting techniques

Fragkakis M. Evangelos[1], Jones E. [2,3], Pountos I.[1], Giannoudis P.V.[1,3]

[1]Academic Department of Trauma and Orthopaedics, School of Medicine, University of Leeds, Leeds General Infirmary, Leeds, West Yorkshire, UK

[2]Leeds Institute of Rheumatic and Musculoskeletal Medicine, University of Leeds, Chapel Allerton Hospital, Leeds, UK

[3]NIHR Leeds Musculoskeletal Biomedical Research Unit, Leeds Teaching Hospital NHS Trust, Chapel Allerton Hospital, Chapeltown Road, Leeds, UK

*Introduction/Objectives*

The clinical applications of Mesenchymal Stem Cells (MSCs) harvested from bone marrow (BM) are well established. Currently, iliac crest (IC) is considered the optimum source, despite known complications. Vertebral body (VB) BM can represent an alternative source promoting bone fusion, especially in spinal operations. Existing literature, comparing ICBM and VBBM MSCs, presents several limitations related to patient cohort, techniques and methodology used. Moreover, the density gradient centrifugation technique (lymphoprep, Lmp) for BM processing is used, despite the effectiveness on ICBM, of the red cell lysis technique (ammonium chloride, AC). This study compared the yield of MSCs between VBBM and ICBM, evaluating the effectiveness of two harvesting procedures and establishing whether MSC behaviour is affected.

*Patient/Methods*

Eighteen patients undergoing elective surgery for idiopathic scoliosis were recruited (median age 15.4 years, range 13-17). BM was aspirated intraoperatively from IC (10ml) and 12th thoracic vertebra (10ml). Aspirates were processed using AC and Lmp. MSCs were enumerated using standard CFU-F assay. Colony areas and MSCs doubling rates were statistically analysed.

*Results*

AC yielded higher leukocyte numbers from ICBM (23x106 cells/ml), compared to VBBM (14.4x106 cells/ml), p=0.013, whereas Lmp yielded similar numbers of mononuclear cells from ICBM (7.5x106cells/ml) and VBBM (5x106cells/ml).

More MSCs could be harvested from 1ml of BM aspirate using AC technique, from both anatomical sites (IC: Lmp 199MSCs/ml, AC 367MSCs/ml, p=0.0123; VB: Lmp 296MSCs/ml, AC 440MSCs/ml, p=0.0425).There were no significant differences in MSC numbers and average colony areas between the sites (IC: AC 14mm2, Lmp 15mm2; VB: AC 11mm2, Lmp 13mm2) or MSCs doubling rates in days (IC: AC 2, Lmp 1.9, VB: AC 1.9, Lmp 1.8).

*Conclusion*

AC results in more effective MSC isolation and does not affect MSC proliferation capacity. VBBM is equally rich in MSCs as ICBM and can represent the first choice for MSC harvesting in spinal surgery.

# Chondrocytes encapsulation in alginate beads: a 3D model for the in vitro production of human cartilage

Galuzzi Marta[1,2], Perteghella Sara[1], Vigani Barbara[1], Grillo Federica[3], Pusateri Alessandro[4], Piepoli Tiziana[5], Montagna Mario[5], Chlapanidas Theodora[1]

[1]University of Pavia, Department of Drug Sciences, Pavia, Italy
[2]Niguarda Hospital, Struttura Semplice Tissue Therapy, Milan, Italy
[3]University of Genoa, DISC, Pathology Section, Genoa, Italy
[4]Fondazione IRCCS Policlinico San Matteo, Department of Otorhinolaryngology, Pavia, Italy
[5]Rottapharm Biotech S.r.L., Monza, Italy

Pre-clinical studies about the aggrecanase inhibitors for the treatment of osteoarthritis have highlighted the limitations associated with the use of the animal model because of the specificity proteases that cut aggrecan with the progressive destruction of cartilage. Tissue engineering is aimed to reconstruct in vitro tissues comparable to those natives, but is difficult finding suitable amount of chondrocytes due to the shortage of donors, the low cell density in the tissues of origin, the loss of cellular phenotype during expansions and the number of culture passages. For these reasons, human nasal septal cartilage was evaluated as a new chondrocyte source and three-dimensional cell cultures were considered to develop a human cartilage model suitable for the screening of new drugs. For this purpose, chondrocytes from two different sources 1) otolaryngology surgery (healthy cartilage of the nasal septum) or 2) from knee surgery (pathological cartilage), were encapsulated in alginate beads at the same density (106 cells/ml alginate), alone or in co-culture with adipose-derived mesenchymal stem cells (ADSCs) in the ratio 30:70 (chondrocytes: ADSCs). Different media were employed to promote extracellular matrix production and differentiation of stem cells, i.e., maintenance medium (with/without growth factors TGF-β1 and/or FGF-2) and differentiation medium (with/without growth factors, dexamethasone and ITS). At different days of culture, total sulfated proteoglycans (GAG) were quantified as a marker of matrix synthesis using dimethylmethylene blue assay (DMB). Results indicate that the culture of nasal septum chondrocytes with TGF-β1 10 ng/ml allows to obtain GAG significantly higher than all other conditions tested, resulting the most promising for the production of a functional tissue in vitro.

# BMSCs and ASCs response to 17 β-estradiol (E2)

Giannasi Chiara[1,2], Niada Stefania[1,2], Ferreira Lorena Maria Josè[1], Arrigoni Elena[1], Brini Anna[1,2]

[1]Università degli Studi di Milano, Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche Milano, Italy

[2]IRCCS Istituto Ortopedico Galeazzi, Milano, Italy

Estrogens confer skeletal protective effects, facilitating osteogenesis and suppressing bone-resorption. 17β estradiol (E2) effect on progenitor cells (MSCs) has been largely studied, nevertheless data are still controversial. Since Mesenchymal Stem Cells from bone marrow (BMSCs) and adipose tissue (ASCs) are widely used in bone-tissue engineering research, we evaluated in vitro the effect of E2 on their proliferation, viability and osteogenic ability. E2 did not influence cell viability and proliferation; in particular, MSCs treated with E2 (1-1000 nM) for 14 days behaved as the untreated ones and no modifications in morphology and proliferation rate was observed after 35 days of treatment. In order to verify a pro-osteogenic effect, MSCs were pre-treated with 10, 100 nM E2 for 7, 21, 35 days and then osteo-differentiated for 14 days. During the osteo-differentiative process E2 increased significantly BMSC ALP activity (+50%), and this marker was further induced by 7 days pre-treatment (+158% 10 nM, +185% 100 nM) and maintained by longer pre-treatments. In contrast, E2 never influenced the osteogenic ability of ASCs at the doses and time points considered. Next, since E2 seems to be involved in telomerase activation, we wanted to evaluate its gene expression and enzymatic activity in our cells. We were unable to detect it by RT-PCR, and its activity was randomly modulated. Our data suggest a difference of E2 treatment on BMSC and ASC response which could be due to the different expression of one of its receptors. We analysed ERĐ expression by western blot and, surprisingly, an ERĐ variant of about 37 KDa is expressed by both BMSCs and ASCs, whereas the classic 66 KDa isoform was faintly or even not depicted. In conclusion, E2 improved osteogenic differentiation just on BMSCs without effect on ASCs; further studies are now in progress to elucidate the ERα variant function in MSCs.

# Neural differentiation of human mesenchymal stem cells from adipose tissue treated with conditioned medium from olfactory ensheathing cells (OECS) or neuroblastoma B104 cell line

Graziano Adriana Carol Eleonora[1], Lo Furno Debora[1], Pellitteri Rosalia[2], Avola Rosanna[1], Giuffrida Rosario[1], Cardile Venera[1]

[1]University of Catania, Department of Bio-medical Sciences, Section of Physiology, Catania, Italy
[2]Institute of Neurological Sciences, National Research Council, Section of Catania, Italy

*Objectives*

The aim of this study was to verify if conditioned medium (CM) obtained from olfactory ensheathing cells (OECs) or B104 neuroblastoma cells was capable of inducing differentiation of mesenchymal stem cells from adipose tissue (AT-MSCs) to a neuronal phenotype. OECs are glial cells of the olfactory nerve axons capable of continuous neurogenesis throughout lifetime in the mammalian olfactory system. They share many phenotypic features of astrocytes and Schwann cells. OECs are a source of multiple trophic factors and seem to play a decisive role in central nervous system plasticity. B104 cells, derived from neuroblastoma, show to possess many neuron-like properties such as electrical excitability and production of neurotransmitters. It was also reported that conditioned medium obtained from B104 neuroblastoma cells (B104-CM) induces oligodendrocyte precursor cells from neural stem cells.

*Materials and Methods*

To achieve our aim, immunocytochemical procedures and flow cytometry analysis were used. Some neural markers, as nestin, protein gene product 9.5 (PGP 9.5), microtubule-associated protein 2 (MAP2), glial fibrillary acidic protein (GFAP), and neuron cell surface antigen (A2B5) were examined 24 hours and 7 days after the treatment.

*Results*

The results showed that both OECs- or B104-CM-treated AT-MSCs express markers of progenitor (nestin), as well as mature neurons (PGP 9.5 and MAP2) in time-dependent manner. They display morphological features resembling neuronal cells, and are immunonegative for GFAP and A2B5, astrocyte and oligodendrocyte markers, respectively.

*Conclusion*

This study demonstrates that AT-MSCs can respond to environmental cues toward a neuronal phenotype. The data obtained might help to develop a human model for replacement therapy in nervous system degenerative diseases. Furthermore, this culture system may offer many advantages, including the ability to perform safety test, to proliferate and store these cells before transplantation.

# Expression of proinflammatory Cytokines by Equine Mesenchymal Stem Cells from foetal adnexa: preliminary data

Iacono Eleonora, Rossi Barbara, Lanci Aliai, Castagnetti Carolina, Merlo Barbara

University of Bologna, Department of Veterinary Medical Sciences, Ozzano Emilia (BO), Italy

MSCs regulate localization, self-renewal and differentiation of hematopoietic stem cells due to MSCs' secretion of cytokines. In the present study, we examined, for the first time in equine foetal MSCs, the expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-8, by RT-PCR analysis. Furthermore, we monitored proliferation and examined CDs expression of MSCs from equine amniotic membrane (AM), Wharton's jelly (WJ) and amniotic fluid (AF). Samples were recovered at labor from 3 mares. Undifferentiated cells, cultured in DMEM-TCM 199, plus 10% FBS, were passaged up to 3 times. Population-doubling times (DT) were calculated, and data were analyzed by ANOVA. No significant differences ($P>0.05$) were found between cell doublings of all passages. DT of AM (2.4±1.0 d), AF (1.8±0.2 d) and WJ (1.9±0.1 d) resulted statistically similar ($P>0.05$). No lag phase was observed during in vitro culture. At P3, characterization was performed. GAPDH was employed as reference gene. Differently from previous studies, all cell lines resulted negative both for MHC-I and MHC-II, confirming the immunosuppressive function of MSCs. AM and AF cells did not express hematopoietic marker CD34 and CD45, but expressed mesenchymal marker, CD90. Differently, WJ cells resulted positive also for IL-8 and weakly positive for CD34. This was already observed in human WJMSCs. Recent reports indicate that CD34+ stem cells have the potential to secrete growth factors, cytokines, and chemokines. However, until now, little is known about direct immune-regulatory functions of CD34+ stem cells during inflammation. All isolated cells resulted negative for CD73. As postulated in a previous study, this could be related to the negative expression of TNF-α and IL-1β in the isolated cells. Further studies are needed to verify if the expression of pro-inflammatory cytokines changes in different culture conditions and at different culture passages, and if their expression could interfere with cells differentiation potency.

# Non-woven silk fibroin mats, adipose-derived mesenchymal stromal cells and platelet lysate for wound healing applications

Perteghella Sara[1], Dotti Silvia[2], Faragò Silvio[3], Boschi Alessandra[3], Tripodo Giuseppe[1], Vigani Barbara[1], Renzi Sabrina[2], Chlapanidas Theodora[1]

[1]University of Pavia, Department of Drug Sciences, Pavia, Italy
[2]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Cell Culture Center, Brescia, Italy
[3]Innovhub, Stazioni Sperimentali per l'Industria, Silk Division, Milan, Italy

In this work, silk fibroin non-woven mats (SF) have been valued in association with adipose-derived mesenchymal stromal cells (ASCs) and platelet lysate (PL) for wound healing applications. Silk fibroin scaffolds were produced with a large scale water entanglement method, sterilized and characterized. ASCs and PL were isolated from adult specific pathogen free New Zealand rabbits. In particular, ASCs were collected from intra-abdominal subcutaneous adipose tissues deposit, while PL was obtained after mechanical disruption of platelet concentrates via -80°C freezing and thawing. SF soaked with PL alone or associated with ASCs were applied on epithelial/dermal wounds carried out on the dorsal surface of animals. Ten animals were divided in 3 groups: group 1 was treated with SF and PL alone, group 2 with SF and ASCs diluted in PL, finally group 3 (control) was treated with SF in physiological solution. A daily observation of the animals was performed (physical examination) and each scaffold was changed every 72 hours, with the same composition and dosage, for a total of 3 applications.
The skin reparative process was solved in 9 days, with a completely restetutio ad integrum of the epithelium in animals treated with PL alone and the regenerative evolution was more rapid than in the animals treated with SF alone. Moreover, the wounds treated with PL alone were characterized by a complete wound re-epithelization with no inflammatory event or proud flesh presence.

# Cell proliferation in 3D cancer spheroids: Volume assessment and 3D reconstruction from a single 2D projection

Piccinini Filippo[1], Anna Tesei[2], Chiara Arienti[2], Serena Duchi[3], Alessandro Bevilacqua[1,4]

[1]Advanced Research Center on Electronic Systems (ARCES), University of Bologna, Bologna, Italy
[2]IRCCS-Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST), Biosciences Laboratory, Meldola (FC), Italy
[3]IRCCS-Istituto Ortopedico Rizzoli (IOR), Osteoarticular Regeneration Laboratory, Bologna, Italy
[4]School of Engineering and Architecture, Department of Computer Science and Engineering (DISI), University of Bologna, Italy

*Objective*
Volume is amongst the most relevant features for the characterization of tumours on a macroscopic scale. Similarly, it becomes the most important feature in pre-clinics, where multicellular spheroids are typically used as 3D tumour models to study the effectiveness of drugs and therapies. Changes in volume are directly correlated to cell proliferation, and a wrong estimation of the volume can jeopardize the assessment of the entire care treatment. Very few methods have been proposed to estimate the tumour volume arising from a 2D projection, such as a widefield microscope image. In this work, we propose Reconstruction and Visualization from a Single Projection (ReViSP), an automatic method conceived to reconstruct the 3D surface and estimate the volume of multicellular spheroids.

*Materials and Methods*
To analyse the different phenotypes we prepared several spheroids by using 3 different cell lines (mesenchymal stem cells, CAEP epidermoid carcinoma cells, and A549 adenocarcinomic epithelial cells) and commercial systems: an RCCS-8DQ bioreactor (Synthecon) and some Perfecta3D (3DBiomatrix) and GravityPLUS (InSphero) hanging-drop plates. Then, we imaged the spheroids by using a confocal microscope. To assess the effectiveness of our method, we compared it with other approaches proposed in literature by using real-world, home-made, macroscopic 3D objects inspired to the spheroid morphology, from which the ground-truth volume can be easily estimated.

*Results*
The estimations obtained by ReViSP were more accurate than those of the other methods tested, with an average volume error lower than 5%. However, the most important finding emerged by our study was that some widely used methods are characterized by an average error higher than 15%, which could completely mislead drug-testing analyses.

*Conclusion*
Our results suggest ReViSP as the most likely candidate for the new gold standard method to estimate spheroid volume. It is worth noting that ReViSP is available as an open-source software tool.

# Assessment of safety parameters in cultured mesenchymal stromal cells for use in regenerative medicine

Renzi Sabrina[1], Dotti Silvia[1], Bonassi Signorini Patrizia[2], Parolini Ornella[2], Ferrari Maura[1]

[1]Centro Substrati Cellulari e Immunologia Veterinaria, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy

[2]Centro di Ricerca E. Menni (CREM), Fondazione Poliambulanza-Istituto Ospedaliero di Brescia, Italy

*Objective*

Aim of this study was the evaluation of several parameters, which could jeopardize the use of mesenchymal stromal cells (MSCs) in cell therapy. Manipulation of cells is considered a potential risk, in fact during this process cells could acquire adverse features, which could compromise MSCs in vivo use. For this reason, it is important to verify safety of cell samples.

*Materials and Methods*

MSCs, isolated from equine adipose tissue (AT) and bone marrow (BM), were amplified for 4 serial passages, controlled for contaminations and characterized by different methods. Self renewal was tested by colony forming unit (CFU) test and multipotentiality was investigated evaluating mesenchymal markers expression and capacity to differentiate into mesodermal lineages. The potential neoplastic/tumorigenic evolution was tested in vitro (soft agar medium, telomerase activity) and in vivo (inoculation into athymic nude mice). Immunomodulatory activity was investigated by testing the capacity to inhibit T cell proliferation.

*Results*

MSCs were successfully in vitro amplified, no batch was positive for microorganism presence. CFU test results indicated that one colony originated by 12000 BMMSCs and 10000 ATMSCs. All samples differentiated into adipogenic, osteogenic and chondrogenic lineages. Flow cytometry assay showed that CD29 expression increased during amplification of ATMSCs, whereas CD44, CD90 and 4E1 expression decreased. MSCs did induce neither neoformation in vivo nor foci of transformed cells in vitro. Telomerase activity analyses of ATMSCs gave rise to variable results. We demonstrated BMMSCs immunomodulatory features.

*Conclusion*

This study demonstrated self renewal and multipotential features of ATMSCs, furthermore they did not demonstrated tumorigenic/transforming potentialities. Contrasting results obtained by telomerase activity, were probably due to sample individual variability. BMMSCs showed self renewal capacity, multipotentiality, immunomodulation properties, whereas they did not demonstrate transforming/tumorigenic features. It appears therefore that following a standardized production process system, the above reported parameters allow to ensure the safety of MSCs.

# Characterization of tendon stem/progenitor cells and in vitro comparison with adipose derived stem cells: a new source for regenerative medicine?

Stanco Deborah[1], Fumagalli Valentina[2], Viganò Marco[1], de Girolamo Laura[1], Perucca Orfei Carlotta[1, 2]

[1]IRCCS Istituto Ortopedico Galeazzi, Orthopaedic Biotechnology Lab, Milano, Italy
[2]University of Pavia, Italy

*Objective*
Adipose tissue and, more recently, tendon are considered promising mesenchymal cell sources for regenerative medicine. Here we compared TSPCs (tendon stem progenitor cells) from human hamstring tendons with human ASCs (adipose-derived stem cells), in order to assess their differentiation potential for possible future therapeutic applications.

*Materials and Methods*
TSPCs and ASCs were isolated from hamstring and adipose tissue of 7 healthy donors. Proliferation, CFU-F ability and stem cell marker expression were evaluated. Multi-differentiation potential was assessed culturing cells in specific differentiation media: alkaline phosphatase (ALP) activity and extracellular calcified matrix deposition were analyzed after osteogenic induction (14 and 21 days, respectively); glycosaminoglycans production was quantified in pellet culture after chondrogenic differentiation (21 days); lipid vacuoles production (Oil-Red-O) was assessed after 14 days of adipogenic differentiation. The expression of osteogenic (RUNX2), chondrogenic (ACAN and SOX9), adipogenic (LEPTIN) and tenogenic (SCX and DCN) genes was assessed by RT-PCR.

*Results*
TSPCs and ASCs showed a fibroblast-like morphology, similar doubling time and clonogenic ability (~10%). Both populations exhibited the typical MSCs surface markers (CD90, CD105, CD73 and CD44) and expressed the stemness-specific transcription factors KLF4 and POU5F1. Both osteo-differentiated cells showed a significant increase of ALP activity levels and extracellular calcified matrix production respect to undifferentiated cells. However, OSTEO-ASCs were able to produce a higher amount of calcified matrix ($p<0.001$) and expressed higher mRNA levels of RUNX2 ($p<0.01$) than OSTEO-TSPCs. Unlike ASCs, CHONDRO-TSPCs showed a significant increase of GAGs levels ($p<0.05$) respect to untreated cells, Undifferentiated and differentiated TSPCs also showed higher ACAN gene expression than ASCs. TSPCs produced higher amount of lipid vacuoles than ASCs after adipogenic differentiation ($p<0.05$). The tendon-related genes SCX and DCN were more expressed in TSPCs compared to the respective ASCs ($p<0.05$).

*Conclusion*
Tendons contain cells able to differentiate into osteogenic and chondrogenic lineages, thus representing a promising sources of MSCs for tissue-engineering based musculoskeletal therapy.

# Web-BI – ICT for stem cell research

Vandoni Lorenzo[1], Breganni Stefano[1], Ciaramella Alberto[2], Ciaramella Marco[2], Perego Gianpaolo[3]

[1]Emisfera soc. coop., Verbania, Italy
[2]Intellisemantic srl, Torino, Italy
[3]Aethia srl, Ivrea, Italy

Web-BI is a research project financed by Piedmont Region, with the goal of automatic extracting from the web information about Stem Cell research, and analyzing it with Business Intelligence techniques.
The research has been directed to solve the following issues:

- Selection of an initial set of affordable web sources
- Automatic detection of new web sources
- Automatic determination of affordability of new sources
- Automatic clustering and digest of documents
- Automatic feeding of data warehouse
- Web access to data warehouse information

Algorithms have been created and prototypes have been built to extract and publish information about clinical trials, enrollments, sponsors, clinical centers, publications and treatments.
Besides, algorithms to find new information sources, determine their affordability, collect documents in clusters, create digest, and convert unstructured information in English language into a structured data warehouse have been implemented and validated.
Starting from these prototypes, the project partners are now building a public web site that will extract information from well-known web sites such as ClinicalTrials and PubMed, and allows for automatic extraction of statistical information at many levels of details.
The web site features are being defined by the project partners in co-operation with the Stem Cell Transplantation and Cellular Therapy Laboratory of the Department of Public Health and Pediatrics - University of Turin.
In the next months it is planned to release an online prototype available to a larger audience.

# Silk Fibroin/alginate scaffolds for the transplantation of pancreatic islets to treat diabetes pathologies

Vigani Barbara[1], Antonioli Barbara[2], Perteghella Sara[1], Pintacuda Domenica Valeria[1], Preda Stefania[1], Faragò Silvio[3], Boschi Alessandra[3], Mastracci Luca[4], Chlapanidas Theodora[1]

[1]University of Pavia, Department of Drug Sciences, Pavia, Italy
[2]Niguarda Hospital, Struttura Semplice Tissue Therapy, Milan, Italy
[3]Innovhub, Stazioni Sperimentali per l'Industria, Silk Division, Milan, Italy
[4]University of Genoa, DISC, Pathology Section, Genoa, Italy

Aim of the present work was to develop a novel therapeutic device for the subcutaneous transplantation of pancreatic islets in type 1 diabetes mellitus treatment: a bimodular scaffold, composed by Stromal Vascular Fraction (SVF) on silk fibroin (SF) non-woven mats and pancreatic islets in alginate beads, was designed. In vivo studies were performed to evaluate device biocompatibility and therapeutic efficacy in a mouse model.

Five mice inbred C57BL/6 (50 days old; 20.11±0.63g) were selected and anesthetized. A SF scaffold (0.5X0.5cm) supplemented with 1*106 SVF, isolated from syngeneic adipose tissues, was implanted subcutaneously in the dorsal area of each animal. After two weeks, diabetes mellitus was induced. Islets were isolated from syngeneic pancreas, purified and encapsulated in alginate beads, which were prepared by extrusion in an isotonic saline solution 50 mM CaCl2 of a cell suspension in 1% w/v sodium alginate solution. Pancreatic islets in alginate beads (~ 525 IEq/15 beads/mouse) were disposed on the surface of vascularized SF scaffold. Mice glycaemia and weight were monitored.

Immunohistochemical analysis showed that SF scaffold induced a typical foreign body reaction, recruiting macrophages and giant cells, but, at the same time, promoted the neo-vascularization of the implanted construct. Already after one day from islet transplantation, one out of five treated animals showed a decrease of blood glucose levels under 200 mg/dL. At day 62, the responder mouse was still normoglycemic and its weight was physiologically increased.

Bimodular device in vivo efficacy was demonstrated: SVF promoted the neo-vascularization of the SF scaffold, allowing that transplanted islets preserved their viability after subcutaneous implantation. Further studies will be conducted using Adipose-derived Stem Cells (ADSCs) instead SVF, in order to investigate the mesenchymal stem cells approach in diabetes mellitus therapy.

# Efficacy of Mesenchymal Stromal Cells therapy for the treatment of idiopathic autoimmune inflammatory diseases of the canine central nervous system

Offer Zeira, Ghezzi Erica, Pettinari Letizia, Konar M., Cantile C., Scaccia Simone, Asiag Nimrod, Aralla Marina

San Michele Veterinary Hospital, Tavazzano con Villavesco (LO), Italy

Granulomatous meningoencephalitis (GME) and necrotizing meningoencephalitis (NME) are common idiopathic autoimmune inflammatory disorders of the canine central nervous system (CNS). The antemortem diagnosis is achieved via assessment of clinical signs, cerebrospinal fluid (CSF) analysis, imaging of the CNS via magnetic resonance imaging (MRI) and infectious disease testing. However, CNS autoimmune inflammation cases could be definitively diagnosed based on histopathological examination. Previous reports have suggested that GME and NME are autoimmune CNS disorders and that suppressing the immune reaction is the best management method for patients. Therefore, several immunosuppressive drugs have been used for autoimmune encephalitis in dogs. Mesenchymal stromal cells (MSCs) are under investigation in clinical trials to treat autoimmune disorders and degenerative disorders due to their immunomodulatory and regenerative properties. Our aim is to verify the safety and efficacy of MSCs treatment in canine idiopathic autoimmune inflammatory disorders of the CNS.

Four bitches presented with neurological signs, such as head tilt, ataxia, circling and paresis were included in the study. In all the patients physical and neurological examinations, magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis were performed. MRI showed intraaxial hyperintense single or multifocal lesions, increased meningeal enhancement, which is unfrequent for the intraaxial lesions. CSF analysis showed typical pleocytosis, mainly monocytic. Autologous bone marrow MSCs (BMSCs) were isolated from each dog, cultured and expanded. In each dog 2x10(6) BMSCs was injected intrathecaly in cisterna magna and 0.5x10(6) BMSCs/kg intravenously. Follow up of 6 and 12 months included clinical evaluation, complete blood and biochemistry work up, neurological evaluation and MRI. MSCs consistently (>98%) expressed their classical surface markers and were negative for lymphocytes and hematopoietic cells (FACS analysis). Neurological evaluation and MRI were normal. BMSCs are a safe and promising therapy for the treatment of CNS-related autoimmune diseases due to their immunomodulatory and neuroprotective effects.

# Mesenchymal Stromal Cells as regenerative treatment in canine advanced liver diseases

Zeira Offer[1], Ghezzi Erica[1], Pettinari Letizia[1], Rossi Federica[3], Rubini Giuseppe[2], Spaziante Daniele[4], Scaccia Simone[1], Asiag Nimrod[1], Martinelli Laura[1], Aralla Marina[1]

[1]San Michele Veterinary Hospital, Tavazzano con Villavesco (LO), Italy
[2]Ultravet Diagnostic, San Giovanni in Persiceto (BO), Italy
[3]Ospedale Veterinarioi Portoni Rossi, Zola Predosa (BO), Italy
[4]FuturaVet, Tolentino (MC), Italy

Mesenchymal Stromal Cells (MSCs) are self-renewing cells, that can be found in almost all postnatal organs and tissues, including liver. Because of their characteristics MSCs are promising therapeutic agents for the therapy of acute and chronic liver diseases.
Chronic liver disease is associated with many serious systemic complications resulting from both liver failure and portal hypertension;
Portosystemic shunts (PSS) are vascular anomalies that divert blood from the abdominal viscera to the heart, bypassing the hepatic sinusoids and carrying intestinal absorption products directly to the systemic circulation and causing an abnormal or diseased state of the liver.
Focusing on the properties that may allow MSCs to treat liver diseases we used autologous MSCs in two cases of extra hepatic PSS and one case of chronical degenerative liver disease.
Autologous bone marrow MSCs were isolated from each dog, cultured and expanded.
During the surgery for the placement an ameroid ring constrictors, the two dogs with PSS received 1,5 x10(6) cells injected in ten different sites under the Glisson capsule.
The dog with the chronical degenerative liver disease received 1,5 x10(6) cells with an eco-guided injection in ten different sites under the Glisson capsule.
Follow up at 3 months included clinical evaluation, blood and biochemistry work up, histological exam of liver biopsy, ultrasound and CT scan for the PSS.
MSCs consistently (>98%) expressed their classical surface markers.
The CT scan for the PSS revealed increase of portal blood flow and liver total volume up to 50%.
Ultrasound for the chronical degenerative liver disease revealed clear increase of portal blood flow.
The histological exams revealed the significantly reduction of steatosis lesions, the proliferative reduction of arteriolar portal proliferation and the augmentation of the portal spaces of the portal veins.
In conclusion MSCs therapy for liver disease has considerable potential.

# General Information

**ORGANIZING SECRETARIAT**
**COGEST M. & C. Srl**
Vicolo San Silvestro, 6
37122 VERONA
Tel. +39 045 597940 - Fax +39 045 597265
E-mail: cogest@tin.it - www.cogest.info

**CONFERENCE & SYMPOSIUM VENUE**
Both events will be held in **Auditorium Banco Popolare**, with access from Viale delle Nazioni, 4 (South Verona).

**ECM - CME (CONTINUING MEDICAL EDUCATION) - Only for Italian participants**
**PROVIDER: COGEST M. & C. - ID 979**

**27th Annual Conference of Italian Association of Cell Cultures (ONLUS-AICC)**
All'evento sono stati attribuiti n. 3 Crediti Formativi ECM per le professioni: **Biologo, Farmacista** e **Medico Chirurgo** (*disciplina:* Oncologia).

**Obiettivo formativo tecnico-professionale n. 18: Contenuti tecnico-professionali (conoscenze e competenze) specifici di ciascuna professione, di ciascuna specializzazione e di ciascuna attività ultraspecialistica. Malattie rare.**

**5th International Satellite Symposium AICC-GISM**
All'evento sono stati attribuiti n. 2 Crediti Formativi ECM per le professioni: **Biologo, Farmacista** e **Medico Chirurgo** (*discipline:* Cardiologia, Neurologia ed Oncologia).

**Obiettivo formativo tecnico-professionale n. 18: Contenuti tecnico-professionali (conoscenze e competenze) specifici di ciascuna professione, di ciascuna specializzazione e di ciascuna attività ultraspecialistica. Malattie rare.**

Per l'attribuzione dei crediti formativi è richiesta la presenza in aula per tutta la durata dell'evento formativo (100%); è necessario aver superato il questionario di apprendimento con almeno il 75% di risposte corrette ed aver compilato la modulistica relativa alla qualità percepita.
**Modalità di verifica della presenza, della qualità percepita e dell'apprendimento**
- Firma di presenza
- Questionario per la rilevazione della qualità percepita
- Test composto da 3 domande per ogni credito attribuito con risposta a scelta multipla

**OFFICIAL LANGUAGES**
27th Annual Conference of Italian Association of Cell Cultures (ONLUS-AICC): Italian
5th International Satellite Symposium AICC-GISM: English

**SECRETARIAT DESK**
The Secretariat Desk at the Conference & Symposium venue will be open:
November 12 from 14.00 to 19.00
November 13 from 8.00 to 18.30
November 14 from 8.00 to 17.30
Secretariat phone during the Conference: 0039 329 4610295

## VERONA PRACTICAL INFORMATION (A-Z)

### Banks

In the city centre there are several banks open from 8.30 to 12.30 and from 14.30 to 16.00 approximately. There are many ATMs 24 hours a day - 7 days a week.

### Cars & Parking

Be careful about driving into Verona city centre because of ZTL areas, which are limited traffic flow areas where the access of cars is limited to pre-established times.

*Free access to the ZTL areas*

Timetable
Monday - Friday:
- from 10.00 to 13.30
- from 16.00 to 18.00
- from 20.00 to 22.00

Saturday, Sunday and holidays: - from 10.00 to 13.30

If staying at a hotel inside a ZTL area, make sure the hotel or its garage calls the plate into the police and obtains a provisional transit permit. Keep the hotel bill in case you need to challenge a fine later.

*Parking*

Free parking in Verona:
- Porta Palio Parking - Stradone Porta Palio
- Piazzale Guardini Parking - Piazzale Guardini
- Stadium Parking (three parks and ride) - Piazzale Olimpia

Pay and display parking:
- Arena Parking - Via Bentegodi, 1 - tel. 0039 045 8009333
- Cittadella Parking - Piazza Cittadella, 4 - tel. 0039 045 595593
- Italia Parking - Corso Porta Nuova, 91 - tel. 0039 045 8006312
- Piazza Isolo Parking - Via Ponte Pignolo, 6 - tel. 0039 045 8007921
- Via Città di Nimes Parking - Via Città di Nimes - tel. 0039 045 2320025
- Arsenale Parking - Via Todeschini, corner Viale della Repubblica - tel. 0039 045 8303460

*Verona Park*

It's a system of payment of parking by purchasing a prepaid parking ticket. The prepaid voucher can be purchased at newsstands, tobacco shops and bars and must be displayed inside the vehicle on the dashboard clearly visible and legible in all its data, after scraping the 5 symbols related to:
- Year
- Month
- Day
- Time (start stop)
- Minutes (start stop)

*Parking meters*

Parking meters are located in the Green Area (ZTL - Limited Traffic Zone) and in the neighbourhoods surrounding the old town Red Area (CORONA AREA): San Zeno, Veronetta, Cittadella, Borgo Trento and Pindemonte. They accept only coins from € 0,10 to € 2,00. Depending on the coins inserted and on the fare in force in the area, they issue a ticket of minimum 30 minutes (€ 0,50 - 30 minutes; € 1 - each hour). The parking meters also offer the possibility of issuing urban bus tickets every day. The ticket issued by the parking meter must be placed on the dashboard of the car. It must be visible by parking enforcement officers.

### Chemist's & Hospitals

In the city centre there are several chemist shops, easy to recognize by a bright green cross.
They open Monday - Friday from 9.00 to 12.00 and from 16.00 to 19.00.
Duty pharmacies open also on weekends and holidays. Find the list of the chemist shops with the timetable on www.farmacieverona.it or outside every chemist shop.

# General Information

Ospedale Civile Maggiore - Piazzale A. Stefani, 1 - Verona
Policlinico G.B. Rossi - Piazzale L.A. Scuro, 10 - Verona
Switchboard 0039 045 8121111

**Credit cards**
In Italy all the main credit cards are accepted.

**Crime and personal safety**
Verona centre is safe, nevertheless we recommend the use of common sense, keeping an eye on wallets when in crowds and possibly avoiding carring too much cash or wearing flashy jewellery.

**Currency**
The Italian currency is Euro (EUR or €).

**Electricity**
Italian voltage is 230 and frequency is 50 Hz. The electric plugs are mainly type L (three pole "Italian" plug). This standard includes two models rated at 10 A and 16 A that differ in contact diameter and spacing. Both are symmetrical, allowing the plug to be inserted at either direction.

**Emergencies**
In case of sanitary emergencies or dangerous situations the number to call is 118
Carabinieri (Italian military police) 112
Police 113
Fire brigade 115

**Getting around**
Verona city centre is extremely rich in historic and architectural beauty; so it is certainly worth a visit on foot. This is the only way to enjoy completely the view of magnificent palaces, squares and monuments that bring us to an imaginary trip back to Verona's glorious history. Moreover, enjoy picturesque glimpses that stimulate your attention and enliven your fantasy!

**Language**
The official language is Italian. Verona is a popular tourist destination, so most of the restaurants and bars staff speak English and German.

**Location and weather**
Verona, located in the north east part of the Italian peninsula, is 59 meters above sea level.
The city experiences hot summers and cold, humid winters, even though tempered by Lake Garda's influence.

**Opening hours**
*Offices:* Monday - Friday 9.00 / 13.00 - 14.00 / 18.30
*Shops:* Monday 15.00 / 19.30 - Tuesday/Saturday 9.30 / 19.30
(some shops open all day from 9.30 to 19.30)
*Supermarkets*: Monday - Friday 8.30 /12.30 - 15.30 / 19.30
In the city centre, supermarkets usually stay open from 8.30 to 19.30 or later.

**Public transport**
Azienda Trasporti Veronesi (ATV) runs urban public transport. Tickets, valid within 90 minutes from the validation, can be bought at tobacconists for € 1,30 or € 1,50 if bought on board. For € 4,00 it is possible to buy a daily ticket, valid 24 hours long on all urban routes.
Here is a summary of the main routes in the city:
· from the station to Piazza Bra (Arena)

Monday - Saturday: buses 11, 12, 13, 72; on Sunday: 90, 92, 93, 96, 97
· from the station to Castelvecchio
Monday - Saturday: 21, 22, 23, 24, 41; on Sunday: 91, 93, 94, 95
· from the station to the meeting venue: bus 61 (ZAI direction), stop Viale delle Nazioni 2.

**Shopping**
Via Mazzini and Via Porta Borsari are appreciated and well known for the great deal of beautiful shops they display.
Footwear, clothes, underwear, leather goods stores, etc. all the best that Italian high fashion labels offer to fashion victims!

**Smoking**
On 10th January 2005 smoking bans were introduced in all the public place, such as bars, restaurants, hotels, offices, shops, etc. with the exception of the places with reserved smoking areas, provided with a suitable ventilation system.

**Taxes**
In Italy all the services and goods are usually subject to VAT tax that is 22% of the value of the goods. In restaurants VAT is 10% and it's usually already included in the bill.

**Taxi**
Radiotaxi switchboard (24 hours a day - 7 days a week) 0039 045 532 666
Taxi stand Piazza Bra 0039 045 803 0565
Taxi stand Porta Nuova Station 0039 045 800 4528

**Telephones**
Italy country code is 0039.
For international calls, dial 00 + national code + area code + personal number

**Time**
The Italian local time is UTC (GMT) + 1, that means one hour ahead of Greenwich mean time. Italy adopts the daylight saving time (DST) from the last Sunday of March till the last Sunday of October.

**Tipping**
In all restaurants, bars, taxis etc. the service is included in the price. Tips are happily accepted but it is not necessary to leave anything more than the amount assessed.

**Verona card**
VeronaCard is an all-inclusive ticket that allows to gain free entry to museums, churches and monuments in the city and travel for free on ATV bus services.
There are two cards available:
- VeronaCard valid for 24 hours (after the first validation) at the price of € 15,00
- VeronaCard valid for 72 hours (after the first validation) at the price of € 20,00
You can buy VeronaCard at museums, monuments, churches, tobacconists and tourist information points in the city centre and at Garda lake surroundings and at all the sales points which participate in the initiative.
For further information: www.veronacard.it

# NOTES

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# VERONA

1. **VERONA PORTA NUOVA RAILWAY STATION**
2. **AUDITORIUM BANCO POPOLARE**
   **Viale delle Nazioni 4, Verona**



ASSOCIAZIONE ITALIANA
DI COLTURE CELLULARI
Onlus

UNIVERSITÀ DEGLI STUDI DI VERONA
Dipartimento Scienze della Vita e della Riproduzione
Sezione di Chimica Biologica



**www.onlus-aicc.org**