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PROCEEDINGS OF THE XXXV NATIONAL MEETING OF THE ITALIAN SOCIETY FOR THE STUDY OF CONNECTIVE TISSUES (SISC)

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European Journal of Histochemistry
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The *European Journal of Histochemistry* was founded in 1954 by Maffo Vialli and published until 1979 under the title of *Rivista di Istochimica Normale e Patologica*, from 1980 to 1990 as *Basic and Applied Histochemistry* and in 1991 as *European Journal of Basic and Applied Histochemistry*. It is published under the auspices of the University of Pavia, Italy.
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Areas of particular interest to the *European Journal of Histochemistry* include:
- functional cell and tissue biology in animals and plants;
- cell differentiation and death;
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- biology of cell development and senescence;
- nerve and muscle cell biology;
- cellular basis of diseases.

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INVITED LECTURES

IDENTIFICATION OF A NEW CELL POPULATION CONSTITUTIVELY CIRCULATING IN HEALTHY CONDITIONS AND ENDOWED WITH A HOMING ABILITY TOWARD INJURED SITES

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With few exceptions, for the repair/regeneration of organ and tissue defects and damages the cell therapy and tissue engineering approaches never really took-off. In addition to still open scientific problems to solve, such as vascularization of large size implants, identification of the "optimal" source of cells and the "optimal" biomaterial carrier, additional bottlenecks are: i) the logistic of collecting from patients, expanding in culture and returning the cells to the surgical theater; ii) the high cost of the culture procedure within the GMP facilities required by the strict rules defined by National and European Regulatory Agencies.

Several decades of stem cell research have progressively uncovered within developed organs a stunning capacity for endogenous regeneration and repair, mediated by specialized stem and progenitor cells residing in their native constitutive tissues. We report on the identification of a rare population of cells present in the peripheral blood of healthy mice that actively participates in the tissue repair/regeneration process. Injury signals are sufficient to (i) specifically direct the recruitment to the wound site of these Circulating Healing (CH) cells; (ii) promote their differentiation and appropriate integration in the regenerative microenvironment. CH cells were identified by an innovative flow cytometry strategy as small cells not expressing CD45 and lineage markers. The analysis of their global transcriptome revealed their uniqueness when compared to other cells characterized by varying stemness degree. Moreover, CH cells presented a high expression of key pluripotency-associated genes and positive selective markers of the epiblast developmental stage.

ANIMAL MODELS TO UNDERSTAND THE PATHOPHYSIOLOGY OF SKELETAL DISORDERS

R. Gioia, R. Besio, S. Maruelli, F. Tonelli, G. Iula, E. Fucci, R. Tenni, A. Rossi, A. Forlino

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Osteogenesis imperfecta (OI), the most common among the heritable skeletal diseases, is characterized by bone deformity, increase fracture rate and growth delay. In the last decay the identification of many different causative OI genes complicated both the clinical and genetic scenario of the disease, making necessary a novel investigation approach. The identification of the molecular mechanisms underlying the various OI forms is particularly useful to identify possible common therapies based on common altered pathways. Up to date, taken into consideration the known abnormal metabolic pathways, we can classify the various OI types in five functional groups, the forms characterized by: 1. Primary defects in collagen structure or processing (with mutations in *COL1A1, COL1A2, BMP1*); 2. Collagen modification defects (due to mutant *CRTAP, LEPRE1, PPIB*), 3. Collagen folding, cross-linking and collagen extracellular matrix interaction defects (due to mutant *SERPINH1, FKBP10, PLOD2, SPARC*), 4. Ossification/mineralization defects (due to mutant *IFITM5, SERPINF1*), and 5. defects in osteoblast development/function with collagen insufficiency (due to mutant *WNT1, CREB3L1, SP7*). The availability of animal models is an indispensable tool to deeply understand the pathogenesis of skeletal dysplasias, being bone a difficult tissue to study directly in humans.

Brtl$^{+/-}$ mice, a murine model for classical OI, allowed us to recognize the relevant role of altered intracellular pathways, caused by mutant collagen type I retention, in a disease thought to be determined only by the presence of abnormal collagen in the bone matrix. This discovery led to the identification of novel potential therapeutic targets for an incurable disease.

Drug screening in mice will be very costly and time consuming, thus a different animal model is necessary.

In the last years, the small fresh water teleost *D. rerio* (zebrafish) imposed itself as a good model for the study of heritable skeletal diseases and several zebrafish mutants have been reported that accurately model human skeletal disorders. Furthermore zebrafish rapid generation time, large offspring number, external development and transparency make it an appealing model for large scale drug screening. Using the *chihuahua* zebrafish OI model already available and generating new ones for the novel OI forms will allow us to test specific molecules targeting the identified alterd intracellular pathways.

OSTEOPOROSIS FROM ETIOPATHOGENESIS TO REHABILITATION

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Osteoporosis is a silent disease until it is complicated by fractures that can occur following minimal trauma.[1] The diagnosis of osteoporosis is established by measurement of BMD. According to the WHO diagnostic classification, osteoporosis is defined by BMD at the hip or spine that is less than or equal to 2.5 standard deviations below the young normal mean reference population[2]. The detoriation of trabecular microarchitecture induced by elevated bone turnover plays an important role in the pathogenesis of osteoporotic fractures[3]. When bone resorption equals bone formation, bone mass remains stable. When resorption exceeds formation, bone mass is reduced-a process that leads to osteopenia or osteoporosis[4]. During a lifetime, a woman will typically lose 50% of her trabecular bone and 35% of her cortical bone.[5] Osteoporotic fractures mainly affect the spine (vertebral crush fractures), leading to loss of height, kyphosis, and chronic back pain; the distal radius (Colles' fracture); and the most serious clinically, the proximal femur ("hip fractures")[6]. Disuse and inactivity can cause bone loss, whereas exercises may maintain or improve bone mineral density. There is a significant correlation between muscle strength and bone mineral density. There is evidence that strengthening exercises may lead to an increase in the mineral density of the bones to which the muscles are attached[7]. Suppression of biochemical markers of bone turnover after 3-6 months of specific antiresorptive osteoporosis therapies, and biochemical marker increases after 1-3 months of specific anabolic therapies, have been predictive of greater BMD responses in studies evaluating large groups of patients[8]. Pharmacologic options for the preven-

tion and/or treatment of postmenopausal osteoporosis include: bisphosphonates, estrogen agonist/antagonist (raloxifene), parathyroid hormone (PTH(1-34), teriparatide) and human monoclonal antibody to the RANKL (denosumab)[9]. It is important to ask patients whether they are taking their medications and to encourage continued and appropriate compliance with their osteoporosis therapies to reduce fracture risk. Physical medicine and rehabilitation can reduce disability, improve physical function and lower the risk of subsequent falls in patients with osteoporosis[10].

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PERSPECTIVES IN CLINICAL PROTEOMICS IN THE LIGHT OF THE COMPLETION OF HUMAN PROTEOME

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The main initiative within the framework of the World Proteomics is the definition of the Human Proteome launched in 2010 is currently achieving its maturity. In the following years a number of associated actions have been opening new routes in the development of new clinical deliverables. These associate the key concepts of Proteomics which links together two fundamental ideas the thorough investigation of protein structure with a functional multifactorial integration. The open unsupervised nature of Proteomics studies have been providing to the scientific community in life science an advance tool to achieve a real novel knowledge of biological phenomena not necessarily link to an a priori hypothesis. These approaches have been bypassing canonical experimental designs which are often based on an arithmetic binary logic. The analogical nature of many molecular objects, in particular proteins, in being part of a large molecular biosystem is a central concept in proteomics investigations. Such an intellectual process is now directly providing a new insight, new ideas to be functionally explored within the fundamental observation of clinical phenotyping. This presentation will introduce and discuss such a problem.

SALIVA: A BODILY FLUID RICH OF INFORMATION

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Saliva is a very attractive bodily fluid for the diagnosis and prognosis of diseases for many reasons: i) collection of saliva is usually economical, "safe", "easy" and can be performed without the help of health care workers (it allows for home-based sampling); ii) it is considered an acceptable and non-invasive process by patients because it does not provoke any pain and so can be easily collected from patients in the pediatric age range[1]. Our group started more than fifteen years ago a study on this bodily fluid with an integrated top-down, bottom-up proteomic platform. The lecture will describe the proteoforms of the main families of human salivary proteins, i.e. proline-rich proteins, histatins, statherins, cystatins, -defensins and -thymosins, characterized by this strategy. Several post-translational modifications occurring before, during and after their secretion will be described, underlying the proteolytic fragmentations from the pre-pro-proteins, which have been characterized thanks to the top-down strategy applied[2].
Even though saliva contains specific families of proteins of secretory origin, they are submitted to post-translational modifications (phosphorylation, sulfation, glycosylation, cyclization, fragmentation) which are due to enzymes common to other exocrine and endocrine glands and tissues[3].
Age related trends will be discussed with a particular concern to the physiological variations observed in pre-term newborns and in the pediatric age range[4]. Some examples of variations of the human salivary proteome observed in multi-factorial diseases will be therefore reported[5].
The putative role in the oral cavity of some salivary proteoforms detected and the demanding issues arising from the proteomic results until now obtained will be finally pointed out.

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THE S100 PROTEINS: PROTAGONISTS OF MANY PHYSIO-PATHOLOGICAL SITUATIONS

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S100 protein family comprises a large group of Ca^{2+}-binding EF-hand proteins. The members are multifunctional proteins only expressed in vertebrates, showing cell-specific expression pattern. S100 proteins may exert both intracellular and extracellular regulatory effects.
Within the cell, S100 proteins are involved in molecular events leading to the regulation of a variety of processes comprising cell proliferation, differentiation, apoptosis, inflammation, and migration through the interaction with several target proteins[1].
Certain S100 proteins can be secreted or released by cells and in the extracellular environment they may exert regulatory activities participating in innate and adaptive immune responses[2]. In particular, it has been shown that S100 proteins are potent modulators of inflammation in the extracellular matrix, and for their role in regulating inflammatory responses these proteins are also classified as DAMPs[3].
Post-translational modifications may orient and modify the biological role of a protein, and for instance it has been observed that oxidation of S100A9 abolished the chemo-repulsive effect on peripheral neutrophils[4].
In the last years, during our investigations devoted to the characterization of proteins/peptides in different biological fluids

under physiological and pathological conditions, we evidenced several S100A proteins, and demonstrated that level of both unmodified and post-translationally modified proteins was affected in a pathology-dependent manner. In this lecture, the major results obtained in the field will be presented and discussed.

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ESTROGEN RECEPTOR ALPHA MEDIATES EPITHELIAL TO MESENCHYMAL TRANSITION, EXPRESSION OF SPECIFIC MATRIX EFFECTORS AND FUNCTIONAL PROPERTIES OF BREAST CANCER CELLS

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The role of extracellular matrix (ECM) the last decades is converted from an inactive network of macromolecules to a functional network essential for structural support, cell migration, adhesion and signaling. Our laboratory has elucidated the significant role of ECM in hormone-dependent breast cancer. ECM molecules, especially proteoglycans (PGs), glycosaminoglycans (GAGs) and matrix metalloproteinases (MMPs), seem to interact with growth factors and receptors in tumor microenvironment by regulating cancer homeostasis. Our recent studies are dealing with novel approaches in respect to the pharmacological targeting at the ECM level in cancer.

Estrogens represent the most important factors implicated in the progression of hormone-dependent breast cancer. The action of estrogens, regulated via estrogen receptor α and β (ERα/β), promotes different roles in tumor initiation and progression. We have recently shown that estradiol (E2) via estrogen receptors (ERs) can regulate the expression of structural and functional extracellular matrix (ECM) macromolecules leading cancer cells to alter their phenotype.

Taking into account the new insights into ER action in breast cancer, we have established in our laboratory a stable cell line MCF-7 SP10+, which has been created after depletion of ERα. Further investigation on this cell line has indicated the significant role of ERα in breast cancer cells homeostasis, via regulation of the expression levels of ECM macromolecules and breast cancer cells functional approaches. Specifically, the expression levels of heparan sulfate proteoglycans have been decreased and on other hand matrix metalloproteinases has been significantly increased. Moreover, the phenotype after depletion of ERα has been changed and the epithelial phenotype switched to mesenchymal.

These data indicated that the role of ERα is of crucial importance for cancer cells behavior and the expression of ECM molecules. Furthermore, might provide a potential target for the design of a more advanced treatment of breast cancer therapies.

This research has been co-financed by the European Union (European Social Fund – ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) Research Funding Program: THALES. Investing in knowledge society through the European Social Fund.

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EXTRACELLULAR MATRIX AS A ROUTE FOR THE ADMINISTRATION OF THERAPEUTIC MOLECULES WITHOUT SECONDARY REACTIONS

L. Messina[1], S. Caruso[1], L. Musumeci[1], S. Bisicchia[1], M. Caputo[1], R. Di Pasquale[1], S. Panfilo[2], P. Mauro[2], S. Vaccaro[1]

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For virtual absence of risk, the oral route is the preferred route of administration of the drugs, but in some situations it is not viable. Intravenous administration may be problematic for example in geriatric patient care and in palliative care, fields in which the subcutaneous administration is a valid alternative. The subcutaneous administration acts in grounds rich in density of blood vessels which allow a systemic spread of the drug. Unfortunately, subcutaneously injected drugs, in order to reach the targets, must go through the interstitial matrix of the skin which limits the quantity and type of therapeutics administered by local injection. In this occasion we could see that a discovered, isolated, CDS cloning, industrial-scale production, highly purified recombinant bacterial (non-pathogenic bacteria strain, *Streptomyces koganeiensis*) hyaluronidase enzyme (rHyal_Sk) depolymerized the viscoelastic component - the hyaluronic acid - of the subcutaneous interstitial matrix in animal models and therefore it increased the dispersion of locally injected high molecular weight drugs. In this way, rHyal_*Sk* improved the pharmacokinetic profiles and significantly increased the total bioavailability of locally injected large protein drugs in systemic blood. What's more, the interstitial hyaluronic acid barriers were restored within 24 h of injection, with neither histologic alterations nor signs of inflammation. rHyal_*Sk* may function as an interstitial delivery boosting agent able to increase the dispersion and bioavailability of co-injected drugs, and all this may lead to the substitution of intravenous delivery with subcutaneous administration of therapeutics in a unprecedented effectiveness.

PRESENTATIONS

PROTEOMIC CHANGES INDUCED BY LOW-INTENSITY ENDURANCE EXERCISE IN MDX MOUSE QUADRICEPS: CORRELATION WITH REDUCTION OF MUSCLE DEGENERATION

S. Fontana[1], O. Schillaci[1], M. Frinchi[2], M. Giallombardo[2], G. Morici[2], V. Di Liberto[2], R. Alessandro[1], G. De Leo[1], V. Perciavalle[3], N. Belluardo[2], G. Mudò[2]

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Previous study showed that low-intensity endurance exercise induced a significant recovery of damaged skeletal muscle in mdx mice, probably by reducing the degeneration of dystrophic muscle[1]. In order to explore the molecular basis of this observation, we perfomed a proteomic analysis to evaluate changes in proteins profiling of quadriceps dystrophic muscles of exercised versus sedentary *mdx* mice. Four protein spots were found significantly changed and were identified as three isoforms of Carbonic anhydrase 3 (CA3) and as superoxide dismutase [Cu-Zn] (SODC). Protein levels of CA3 isoforms were found significantly up-regulated in quadriceps of sedentary *mdx* mice (MDX-Sed) and were completely restored to wild type values in quadriceps of exercised *mdx* mice (MDX-Ex). Protein levels of SODC were found down-regulated in quadriceps of sedentary *mdx* mice and were significantly restored to wild type values in quadriceps of exercised *mdx* mice. These proteomic data, validated by Western blot analysis, indicate that low-intensity endurance exercise, by modulating some proteins involved in oxidative stress defense, may in part contribute to reduce the reported cell degeneration in *mdx* muscles[1].
Further investigations are needed to better define the extension of proteins change in MDX-Ex versus MDX-Sed mice and its correlation with the recovery of damaged fibers in MDX-Ex mice.

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ALTERED CYTOSKELETAL ORGANIZATION MODULATES THE PHENOTYPIC VARIABILITY IN A MURINE MODEL OF OSTEOGENESIS IMPERFECTA

R. Besio[1], S. Maruelli[1], R. Gioia[1], F. Tonelli[1], L. Bianchi[2], A. Gagliardi[2], L. Bini[2], K.M. Kozloff[3], B.M. Khoury[3], J.C. Marini[4], A. Rossi[1], A. Forlino[1]

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Osteogenesis imperfecta (OI) is a heritable bone disease characterized by a wide spectrum of clinical outcomes ranging from very mild to lethal. Identical molecular defects are associated in OI with phenotypic variability, a recurrent feature in hereditary diseases. Brtl$^{+/-}$ mice, a model of dominant OI, show either a moderately severe or a lethal outcome associated with the same Gly349Cys substitution present in the 1 chain of type I collagen, thus they are a valid tool to investigate the molecular basis of OI phenotypic variability. Our previous data revealed that the intracellular machinery in lethal mice is less effective at coping with the intracellular retention of mutant collagen favoring up-regulation of molecules involved in apoptosis with respect to the mice with a moderately severe outcome in which chaperone up-regulation is predominant[1,2]. Here we demonstrated by immunohistochemistry with fluorescent phalloidin, a specific marker for actin filaments, the presence of an abnormal cytoskeleton in calvarial bone, long bone, skin and lung in Brtl$^{+/-}$ mice with lethal outcome (ML). In the same tissues in the ML mice we detected also a reduced number of integrin-based focal adhesions (FAs). In long bone of ML mice collagen deposition was impaired as well as TGF- signalling and ML calvarial osteoblasts revealed reduce cell proliferation as well as decrease expression of the early osteogenic marker Runx2. Thus in ML animals alterated actin filaments and FAs negatively affects cell differentiation, extracellular matrix composition, cell signaling and cell–matrix interaction. The consequence of this dysregulation have an impact on the bone structural properties: ML bones showed significantly reduced lenght, BV/TV and cortical thickness with respect to wild type both by histomorphometric and nanoCT analysis. Importantly, abnormal cytoskeletal assembly was detected in fibroblasts obtained from lethal, but not from non-lethal, OI patients carrying a substitution at the same glycine[3]. Our study identify cytoskeleton as a phenotypic modulator and as a potential novel target for OI treatment.
The work was supported by Fondazione Cariplo [grant n. 2013-0612], Telethon [grant n. GGP13098] and the European Community, FP7, "Sybil" project [grant n. 602300].

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IN VITRO EFFECTS OF OF AgNPs EXOPOLYSACCHARIDE FROM *KLEBSIELLA OXYTOCA* DSM29614 ON BREAST CANCER CELLS

M. Buttacavoli[1], NN. Albanese[2], G. Gallo[1], AM. Puglia[1], C. Faleri[3], M. Gallo[4], F. Baldi[4], S. Feo[1], P. Cancemi[1,2]

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Bacterial exopolysaccharides (EPSs), high-molecular-weight sugar polymers surrounding bacterial cells , have achieved considerable attention because of their potential applications in many fields, including biomedicine, exspecially as antineoplastic molecules. A *Klebsiella oxytoca* DSM 29614 (KO) strain, ex BAS-10, produces an EPS made of rhamnose, glucuronic acid and galactose, which shows metal-binding properties[1,2]. More recently, it has been reported that KO in the presence of $AgNO_3$ is able to synthesize Ag nanoparticles (AgNPs) embedded in branched EPS (AgNPs-EPS). The AgNPs-EPS, produced under aerobic and anaerobic conditions, contain Ag^{+1} and Ag^0 that could have different biological activity[3]. The present study was aimed to assess the cytotoxic effects of AgNPs-EPS, produced under aerobic and anaerobic conditions, on breast cancer cell line SK-BR3. The responses to the AgNPs-EPS treatments revealed a dose dependent behavior resulting at 5 g/ml in a inhibition of cell proliferation rate of 50% (IC50), dramatic

morphological changes consistent with apoptotic features and extensive proteomic modulation . The most important effects were obtained by aerobically biosynthesized AgNPs-EPS treatment, due to the major release of Ag^{+1}, as verified by voltammetry analysis. Proteomic analysis showed modulation of several proteins related to oxidative stress and apoptotic and mitochondrial pathways. Taken together, these results provide new important elements in support of the potential antitumoral activity of AgNPs-EPS.

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COMPARATIVE PROFILING BY PROTEOMICS AND ZYMOGRAPHIC ACTIVITIES OF TUMORAL AND NON-TUMORAL CELL LINES

R. Musso[1], G. Di Cara[1], N.N. Albanese[1], P. Cancemi[1,2], D. Martini[3], C. Giordano[1,4], I. Pucci Minafra[1]

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The extracellular matrix (ECM) underlying epithelial tissues is involved in the maintenance of cell polarity and homeostasis. ECM is a dynamic structure under the regulated remodeling of its components. The major enzymes responsible of matrix degradation are the matrix metalloproteinases (MMPs), a well known family of zinc-dependent endopeptidases. Much attention has been focused on MMP-2 and MMP-9 because of their ability to degrade type IV collagen, a major constituent of basement membranes.

A deregulated proteolysis of ECM molecules may cause the alteration of cell polarity and may contribute to the disruption of cell–cell and cell–ECM adhesions, promoting cancer progression. These alterations are responsible for a poor prognosis, and a positive correlation between the increase of MMPs and the degree of malignancy has also been observed FOR many tumor histotypes. To approach these issues on in vitro models, we performed a comparative study, between a couple of tumoral and non-tumoral mammary cell lines and a couple of thyroid cell lines derived respectively from a benign and malignant cancer.

This experimental approach, based on scanning electron microscopy, on proteomic analysis and on gelatin zimography, highlighted a similar profiling of the two differential couples of cell lines: that is between malignant and non-malignant cells respectively, regardless of their histological origin.

In particular, it was observed that the cell lines derived from aggressive cancers, when compared with their non-malignant counterpart, showed an increased secretion of MMPs, a cell shape highly pleomorphic and a higher expression of protein clusters potentially associated with invasion and metastasis. The analysis of the interactions between the expression of MMPs and of selected proteomic clusters have offered important indication on the complex network existing between neoplastic cells and their environment.

The work co-funded by the Italian 5x1000 to COBS.

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEINS IN ATHEROSCLEROTIC PATIENTS WITH TYPE 2 DIABETES

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Atherosclerosis is a form of chronic inflammation characterized by the accumulation of lipids and fibrous elements in medium and large arteries that represents a major cause of death and disability in people with diabetes.

The aim of this study is to identify differentially expressed plasma proteins between patients with or without type 2 diabetes undergoing carotid endarterectomy, by applying two-dimensional electrophoresis analysis coupled with mass spectrometry.

Briefly, 14 plasma samples from diabetic patients and 15 plasma samples from non-diabetic patients were subjected to a low-abundance proteins enrichment step using hexapeptide combinatorial ligand libraries (ProteoMiner™ enrichment kit, Bio-Rad Laboratories) followed by two-dimensional electrophoresis. This analytical technique allows resolving hundreds of different protein isoforms according to both isoelectric point and molecular weight. Protein profiles were compared by using PD-Quest software (Bio-Rad Laboratories) and spots of interest were identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS). Differential analysis was validated by 1D- and 2D-western blotting. An interaction map was made using String 10 (http://string-db.org/).

A panel of proteins differentially expressed between the two groups of atherosclerotic patients have been identified. Among them, there are fibrinogen beta and gamma chains, complement c1r, c3 and c4-B subcomponents, alpha-1-antitrypsin, vitronectin and some apolipoproteins. Preliminary results on predicted protein-protein interactions suggest that vitronectin could play a role in modulating fibrinolysis, complement dependent immune responses and other pathways in diabetes. Actually, identification of markers in diabetic patients could be of interest for clarifying the biochemical mechanisms underlying the strong association between diabetes and atherosclerosis.

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EXTRACELLULAR VESICLES SHED BY A375 MELANOMA CELLS, CONTAIN H1° RNA AND RNA-BINDING PROTEINS

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Extracellular vesicles (EVs) are shed in the extracellular environment by both prokaryotes and eukaryotes. Although pro-

duced from both normal and cancer cells, malignant cells release a much higher amount of EVs, which also contain tumor-specific proteins and RNAs.
We previously found that G26/24 oligodendroglioma cells shed EVs that contain the pro-apoptotic factors FasL and TRAIL[1,2]. Interestingly, G26/24 release, via EVs, extracellular matrix remodelling proteases[3], and H1° histone protein[4], and mRNA. To shed further light on the role of EVs in discarding proteins and mRNAs otherwise able to counteract proliferative signals, we studied a melanoma cell line (A375). We found that also these cancer cells produce H1° and release it into the extracellular space by EVs. Interestingly, H1° sorted to vesicles has a molecular mass higher than expected, and is probably sumoylated. By T1 RNase-protection assay with the H1° RNA, three main complexes were evidenced in EVs, the most abundant of which has a molecular mass of about 65 kDa. By using a biotinylated H1° RNA to fish interacting factors, we isolated from EVs a few proteins which have been then identified by mass spectrometry: the most abundant is a protein of about 60 kDa: myelin expression factor-2 (MYEF2). Western blot analyses confirmed the presence of MYEF2 in EVs released from A375 melanoma cells.

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DECORIN EFFECTS ON PROTEOMIC PROFILING OF BREAST CANCER CELLS: AN UPDATED STUDY.

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The malignant carcinomas are characterized by several capabilities acquired by the neoplastic cells, among which the ability to invade the extracellular matrix (ECM) and to establish a crosstalk with several ECM components.
Under this respect, the extracellular microenvironment is an entity extraordinarily rich of information with opposite signals. Our group has long undertaken the study of the effects of ECM molecules on the behavior of cancer cells in vitro. Among the studied molecules, the decorin was found to exert a non-permissive effect on the growth and motility of the transfected tumor cells. The decorin, belongs to the family of small leucine-rich proteoglycans (SLRP) and is involved physiologically in the fibrillogenesis of collagen. In the last few year, a new anti-oncogenic role has been proposed for decorin[1].
This study aimed to implement the knowledge on the effects of ectopic decorin on breast cancer cells, using as a reference point the results already achieved by our research group[2] on the experimental model format. By breast cancer cell line 8701-BC and its transfected clone DEC-C2.
The extension of the proteomic analysis combined with the mass spectrometry, allowed to triplicate the number of identified proteins in our model. Among the newly identified proteins were members of the classes of metabolic enzymes, S100 family and cell motility proteins, which revealed a net decrease in the decorin transfected cells. Of considerable importance is the observation that these classes of proteins are the most involved in metastatic progression. These results confirm and reinforce the anti-oncogenic role hypothesized for decorin.
The work was co-funded by the Italian 5x1000 to COBS.

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HIGH MOLECOLAR WEIGHT HYALURONAN MODULATES SERGLYCIN-MEDIATED CD44 ACTIVATION IN CHONDROCYTE CULTURES STIMULATED WITH IL-1β

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Serglycin is a widely distributed proteoglycan, previously assumed to be hematopoietic cell specific. However, during the last decade, numerous studies have demonstrated that such PG is also synthetized by various non-hematopoietic cell types and it is involved in a plethora of both normal and pathological conditions. Serglycin secretion could be induced in several cell types upon external inflammatory stimulation. The biosynthesis of serglycin is up-regulated by lipopolisaccaride (LPS) in macrophages and in primary human endothelial cells, by tumor necrosis factor (TNF) in endothelial cells and adipocytes, as well as by IL-1β in smooth muscle cells. Our data show that serglycin is also synthetized in primary human chondrocytes following stimulation with IL-1β. Since serglycin has been shown to be a ligand for hyaluronan receptor CD44 and such interaction could amplify inflammatory process, we decided to evaluate the mRNA and protein levels of CD44 after IL-1β administration in chondrocytes. The stimulation of cells with IL-1β resulted in an increase of both serglycin and CD44 mRNA and protein expression. Therefore, we observed a significant increase of pro-inflammatory cytokines, such as TNF-α and IL-6. These results suggest that serglycin, as well as CD44, could participate in the inflammatory process of chondrocytes. To further analyze the importance of serglycin during inflammatory response in human chondrocytes we treated such cells with a serglycin siRNA in order to block its production. Blocking serglycin production caused a significant reduction of CD44, as well as of the pro-inflammatory cytokines levels, indicating that the serglycin, released following IL-1β stimulation , is able to increase inflammation by modulating CD44 activity in human chondrocytes. The treatment with high molecular weight hyaluronan (HMWHA), after IL-1β administration, induced a further decrease in pro- inflammatory cyotokines and also a reduction of serglycin production. The effect was more marked when both serglycin siRNA and HMWHA were added together. Such results, taken together, suggest that this proteoglycan is able to modulate inflammation via CD44 receptor. In conclusion, we believe that the serglycin pathways should be carefully considered for future anti-inflammatory strategies although further studied are needed to fully confirm these findings.

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MULTIOMICS ANALYSIS OF S100 PROTEINS IN BREAST CANCER

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The S100 gene family is the largest subfamily of calcium binding proteins of EF-hand type, expressed in tissue and cell-specific manner. S100 proteins act as intracellular regulators and as extracellular signaling. Within cells, S100 have been involved in the regulation of proliferation, differentiation, apoptosis, energy metabolism, inflammation, migration and invasion via interactions with a variety of target proteins. Extracellular S100 proteins act in an autocrine and paracrine manner through the activation of surface receptors that regulate cell proliferation, differentiation, survival and migration. More recently, there is growing interest in the S100 proteins and their relationship with different cancers because of their involvement in a variety of biological events closely related to tumorigenesis and cancer progression[1]. However, the occurrence, the role and the possible coordination of this group of proteins in breast cancer is still poorly known. We previously describe a large-scale proteomic investigation performed on breast cancer patients for the screening of multiple forms of S100 proteins[2,3]. Our results have shown that the majority of S100 proteins are preferentially expressed in the tumor mass compared with the normal adjacent tissue and that some S100 protein members were ubiquitously expressed in almost all patients, while others appeared more sporadic among the same group of patients. More interestingly, patients which developed distant metastases showed a general tendency of higher S100 protein expression, compared to the disease-free group. Present study was aimed to assess the gene expression levels of the S100 protein family members utilizing a breast cancer dataset generated on Affymetrix microarrays technologies[4]. GOBO (Gene expression-based Outcome for Breast cancer Online) is a user-friendly online tool that allows, also, the identification of co-expressed genes and association with outcome in an 1881 breast cancer samples. Other important association with breast cancer outome was carried out by Kaplan Meir-plotter database[5]. Integrating results obtained by proteomic and trascriptomic analysis of S100 proteins highlight their important involvement in breast cancer progression, and support the idea that S100 proteins are important prognostic factors, related to survival period of tumor patients. However, the specific mechanisms by which S100 proteins affect progression of breast require further study.

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CURCUMIN REDUCES INFLAMMATORY EFFECTS EXERTED BY 6-MER HYALURONAN IN HUMAN CHONDROCYTES

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Hyaluronan (HA) fragments produced during many pathological conditions may contribute to amplify pro-inflammatory response during tissue injury[1]. HA oligosaccharides may enhance an inflammatory response by interacting with the toll-like receptor 4 (TLR-4), toll-like receptor 2 (TLR-2) and CD44. The TLRs activation triggers a pathway that leads to the nuclear translocation of the transcriptional nuclear factor kappaB (NF-kB), that in turn induces the expression of different inflammatory mediators[2].
Curcumin (diferuloylmethane) is a phytochemical with anti-inflammatory and anti-oxidant properties. It has been shown to have suppressive effect on NF-kB signaling pathway in various cell types, including condrocytes[3].
The aim of this study was to investigate the effect of curcumin treatment in a human chondrocyte cell line stimulated with 6-mer hyaluronan oligosaccharides.
6-mer HA treatment induced up-regulation of CD44, TLR4 and TLR-2 mRNA expression and related protein levels, and NF-kB activation, that in turn increased iNOS, IL-1beta, IL-6, MMP-9 e MMP-13 expression. Treatment with curcumin decreased NF-B activation and pro-inflammatory mediators, while had no effect on CD44 and TLRs activation.
These data showed that curcumin is able to reduce pro-inflammatory effect induced by HA oligosaccharides in chondrocytes. Since it has been suggested that HA fragments contribute to develop joint inflammation and cartilage damage in rheumatoid arthritis, curcumin may could be beneficial in the management of this chronic disease as a suitable adjunct to conventional pharmaceutical therapy.

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EFFECTS OF CANCER AND STROMAL CELLS CROSSTALK ON HYALURONAN AMOUNT IN AN *IN VITRO* TUMOUR ENVIRONMENT

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Extracellular matrix (ECM) is a complex network of macromolecules and secreted factors that ensures the tissue integrity and physiologic properties by modulating the hydration and osmotic balance in the tumor microenvironment. It is well known that dysregulation of the composition of the ECM is associated with several pathologies, such as breast cancer[1]. Among various ECM glycosaminoglycans, hyaluronan (HA) has a remarkable structural importance[2] but also a role in regulating cellular processes through a binding with membrane receptors and activation of signalling pathways. The role of HA in tumour cells' functions depends on its molar mass. Moreover, matrix with high amount of HA around tumours favour the cancer cells migration and infiltration of newly formed blood vessels[3]. At all stages of tumourigenesis, stromal cells become "activated" and release growth factors and cytokines that further increase HA synthesis in both stromal and tumour cells.
In our laboratory we performed studies on the cross-talk between tumour and surrounding stroma using co-culture sys-

tem (Transwell). We discovered a new protein in the conditioned medium of the low invasive breast tumour cell line BC8701, called "Uncharacterized protein of c10orf118" or "Q7z3e2". Further studies demonstrated that Q7z3e2 protein was mainly expressed and synthesized by tumour cells. Q7z3e2 is mainly localized around or within the nucleus. Then, we show that a Q7z3e2 increase protein level in breast cancer cell lines is correlated to an up-regulation of HAS2 mRNA, as well as to an increase of pericellular and secreted HA in stromal cell line. In order to study the effect of this protein on HA amount in tumour environment, we overexpressed this protein in MCF-7 and we directly co-cultured the cells with normal skin fibroblasts. It was noticed an increase of secreted HA found mainly around tumour cells colony and in contact with fibroblasts. To demonstrate that it is the protein that provokes this HA increase, fibroblasts were treated with a recombinant protein of Q7z3e2 and an increase of HAS2 mRNA was observed.
Considering the above data, these results suggest that breast cancer cell lines synthesize a novel and uncharacterized soluble protein called Q7z3e2 that up-regulates HAS2 mRNA but also increase the extracellular HA amount synthesized by fibroblasts.

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REMOVAL OF α-GAL XENOGENIC BARRIER FROM BIOPROSTHETIC HEART VALVES: TOWARDS IMPROVING LIFE EXPECTANCY OF YOUNGER CARDIOPATHIC PATIENTS

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Every year, 300.000 patients are receiving bioprosthetic heart valve or pericardial patches, both of bovine or equine origin. Heart valve replacement constitutes a major healthcare problem worldwide (the second most frequent cardiac surgery procedure). Bioprosthetic heart valves suffer from late dysfunction restricting their successful application to older recipients. Moreover re-interventions weigh heavily on the economic and social aspects, undermining the quality of life of the patient.
The primary cause of failure of bioprosthetic heart valves is dystrophic calcification, strongly related to the chronic inflammatory/immune reactions elicited by residual exposed xenoepitopes.
Xenogeneic tissues are widely employed in cardiac surgery, but in the absence of any assessment of the extent of xenogeneic epitope removal. The well known glutaraldehyde treatments are reducing but not eliminating the immunogenicity, particularly for the α-Gal epitope beyond granting a complete immuno-tolerance.
We are working on and have developed an experimental procedure allowing to remove the α-Gal xenoantigen. A significant potentiality of this treatment is its application to current bioprostheses without introducing any major change in the production process, while ensuring an important containment of the industrial production costs.
The procedure has been devised in order to process bioprosthetic heart valves currently available on the market (Magna model, Edwards Lifesciences, shown) and equine pericardial patches (XAG-400 model, Edwards Lifesciences, shown). The amount of α-Gal epitopes before treatment, expressed as number of epitopes / 10 mg of wet tissue, accounted to 5.21± 0.7*10^{10} and 9.7 4.9*10^{10}, respectively. After the treatment the α-Gal antigens were completely removed as also confirmed by immunofluorescence analysis.

THE ROLE OF TENDON WATER AND TENDON SHEATHS IN TENDON BIOMECHANICS

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Tendon transmits forces from muscle to bone to promote joint movements. Collagen, the main component of the dried tendon, shows a hierarchical arrangement from a molecular to a supramolecular level (tropocollagen, microfibril, fibril and fibre) with an alternating handedness increasing the resistance of fibrils to tension and ensuring a rotation stability. When Achilles tendons of rats were stretched in vivo and then observed at the transmission electron microscope to measure the fibrils diameter and interfibril spaces, the fibril diameter and interfibril spaces showed a reduction of 18% and 50% respectively when compared to relaxed tendons. From a mechanical point of view this may be explained comparing a fibril to a right handed rope that undergoes to a shrinkage when it is stretched. However the 60-70% of the total weight of tendon is represented by water. Collagen fibres and hydrophilic proteoglycan aggregates (such as decorin, biglycan, aggrecan and versican) retain the main part of bound and free water, respectively. Free water shows a radial extrusion along the outside surface of the tendon when tendon is stretched[1,2]. Tendon sheaths like epitenon and paratenon have been described at the outside surface of Achilles tendon[3]. Wide spaces between the inner epitenon and the outer paratenon were observed. Between these two tendon sheaths a mesotenon connecting and at the same time spacing out the epitenon from paratenon was described at the scanning electron microscope. The wide spaces delimited by mesotenon, epitenon and paratenon could contain the amount of water radial extruded during tendon stretching. The elastic properties of endotenon, paratenon and mesotenon and the orientation of the fibres in the these sheaths could favour the return of the water from the outer portion of tendon to the inner one, also helping the crimping system[4] in recoil of tendon fibres when the tendon relaxes after stretching.

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CANT1 AND PROTEOGLYCAN METABOLISM: AN I*N VIVO* APPROACH WITH MOUSE MODELS OF DESBUQUOIS DYSPLASIA TYPE 1

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Desbuquois dysplasia (DBQD) is a recessive osteochondrodysplasia characterized by growth retardation, short stature, joint laxity and advanced carpal ossification. Two types of DBQD have been described depending on the presence (type 1) or absence (type 2) of typical hand anomalies including additional carpal

ossification centers and delta phalanx. DBQD type 1 is caused by mutations in Calcium-Activated Nucleotidase 1 (CANT1), while DBQD type 2 by mutations in xylosiltransferase 1 (XYLT1).
CANT1 is a nucleotidase present in the ER/Golgi that preferentially hydrolyzes UDP, suggesting its involvement in protein glycosylation and in proteoglycan metabolism.
To better investigate CANT1 role in the etiology of DBQD, we generated a Cant1 knock-in (KI) mouse carrying the R302H substitution reproducing the R300H mutation already observed in patients and a Cant1 knock-out (KO) mouse by excision of exon 3 and 4.
Double staining with alcian blue and alizarin red demonstrated that KI mice are smaller with shorter and thinner tibiae, femurs and ilia compared to the wild-types. In limb extremities of KI mice the same hand anomalies present in DBQD patients were observed. At the morphological level KO mice showed growth defects and typical skeletal anomalies already observed in KI mice and patients. These results demonstrated that both mouse strains develop a skeletal phenotype reminiscent of DBQD type 1. Proteoglycan (PG) metabolism was studied in chondrocytes from KO mice. To study PG synthesis, chondrocytes were labeled with 35S-sulfate and the amount of newly synthesized PGs was evaluated: reduced synthesis of PG was observed in KO chondrocytes compared to wild-type cells both in presence or in absence of β-D-xyloside, an enhancer of glycosaminoglycan (GAG) synthesis. Moreover, the hydrodynamic size of GAGs was investigated by gel chromatography demonstrating that GAGs from KO chondrocytes were smaller in size than wild-type ones. Pulse-chase labeling of chondrocytes showed reduced PG secretion in KO cells compared to wild-types. This result was confirmed by electron microscopy demonstrating the presence in KO chondrocytes of huge vacuoli containing proteinaceus material.
In conclusion we generated and validated a KI and a KO mouse as animal models of Desbuquois Dysplasia type 1 and we demonstrated that CANT1 plays a role in PG metabolism.
This work was supported by Telethon-Italy (grant n. GGP11079) and the European Community (FP7, "Sybil" project, grant n. 602300).

DIFFERENTIAL INFLUENCE OF HYPOXIA ON GENE EXPRESSION OF TUMORAL AND NON TUMORAL MAMMARY CELLS

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Cancer metastasis is the result of a series of deregulated biological phenomena, including alterations of cell-cell and cell-matrix interactions and of other microenvironmental conditions such as the oxygen tissue supply. Hypoxia is a well-known driver of aggressive cancer phenotypes, indeed tumors with poor prognosis have higher proportions of anoxic and hypoxic areas[1]. The consequences of tumour hypoxia can be local or even systemic towards distant organs, and it can evoke diversified responses: whereas low *oxygen concentration in tissue* environments. (pO_2 <7 mmHg) exerts anti-proliferative effects and promotes differentiation, apoptosis and/or necrosis on normal cells, the tumoral cells react to hypoxic stress with adaptive processes that confer them an aggressive phenotype[2]. Indeed, the hypoxic microenvironment in tumors contributes to alter energy metabolism, cell growth and responsiveness to therapy.
The aim of present study was the identification, by a proteomic strategy, of the effects exerted by hypoxic conditions on the 8701-BC breast cancer cells compared with HB2 immortalized normal mammary epithelial cells. For this purpose, the two cell cultures, were grown at low oxygen content (pO_2=2%) in parallel with normoxic cells (pO_2=20%).
Hypoxic and normoxic cells at confluence were then properly collected, lysed and subjected to 2D-IPG based proteomic analysis[3]. Proteins identified by several methods[4] were then clusterized by using the gene ontology database DAVID. The results showed that the hypoxic condition exerts different effects on the proteomic profile of the two cell lines.
In particular, a general down-regulation of the proteome complement was observed for the HB2 cells and especially for the classes of the negative regulators of apoptosis and of the proteins involved in membrane vesiculation. Conversely, the proteomic profile of the 8701-BC cells was not altered significantly by the hypoxia, except for the highly modulated protein class of the cytoskeleton.
These data suggest that hypoxia may depress cell behaviour of non-tumoral cells, while is ineffective on neoplastic cells, basically adapted to anaerobic metabolism, or even promotes cell motility which contributes in directing the tumour cells to acquire a more aggressive phenotype.

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HIPK2 AND HEPARANASE : NEW PLAYERS IN RENAL FIBROSIS

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characterized by the accumulation of extracellular matrix that when persists can lead to organ failure. A key event in tubulointerstitial fibrosis is the epithelial-to-mesenchymal transition (EMT) of tubular epithelial cells into myofibroblasts. In the kidney, EMT is triggered by several factors: hypoxia, reactive oxygen species, advanced glycation end products and numerous profibrotic cytokines and growth factors such as FGF-2 and TGF-β. Homeodomain-interacting protein kinase 2 (HIPK2) is a conserved serine/threonine nuclear kinase that regulates gene expression by phosphorylating transcription factors and accessory components of the transcription machinery. The dysregulation of HIPK2 can result in p53 dysfunction and augmented proliferation of cell population as it occurs in cancer and fibrosis. Recently, it has been shown that HIPK2 is a master regulator of kidney fibrosis in experimental models of chronic kidney diseases (CKD)[1].
Several experimental data also support the involvement of heparanase (HPSE) in the pathogenesis of kidney fibrosis. HPSE is an endoglycosidase that cleaves heparan sulfate (HS) chains and participates in ECM remodeling and degradation as well as in the regulation of the release from ECM storages of HS-bonded molecules. Recently we provided evidence that HPSE is specifically involved in the establishment of tubular fibrosis, being necessary for the epithelial-mesenchymal transition (EMT) of tubular cells induced by FGF-2 and TGF-β[2,3]. Starting from these evidences, we aimed to characterize the role of HIPK2 as fibrosis regulatory molecule and the possible link between HIPK2 and heparanase in pro-fibrotic condition. In

particular we aim to analyze the effect of HIPK2 depletion in the expression of EMT and fibrosis markers in human fibroblast (hFB) and kidney tubular epithelial (HK2) cell lines. Preliminary RT-PCR analysis performed in hFB HIPK2-depleted cells showed the induction of HPSE and EMT markers such as vimentin, N-cadherin and αSMA, while the e-cadherin was down-regulated. The results of this research project could identify the interplay between HIPK2 and HPSE as a novel therapeutic target against the development of fibrosis.

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DONOR'S AGE AFFECTS MINERAL DEPOSITION IN CULTURED DERMAL FIBROBLASTS

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During ageing, ectopic calcifications can be observed in many inherited as well as age-related disorders affecting soft connective tissues. Never the less, only few studies have been performed to better understand if human dermal fibroblasts (HDF) play a role in the development and progression of pathologic mineralization and if they become more responsive to potential calcifying stimuli with age. It has been demonstrated that aging is associated, for instance, with progressive accumulation of sublethal damages, with qualitative and quantitative changes of extracellular matrix production, with altered redox balance, thus causing epigenetic modifications and influencing cells' behaviour. We have recently demonstrated that HDF, when cultured *in vitro*, at least for some parameters, exhibit different features depending on donor's age. Moreover, the observation that altered phosphate regulation can be responsible for ectopic calcification in premature ageing syndromes, further sustains the relationship between ageing and aberrant mineralization and underlines the importance of pyrophosphate (PPi)/phosphate (Pi) balance in these events, where PPi and Pi act as an inhibitor and a promoter of hydroxyapatite crystal growth, respectively.

The aim of the present study was to evaluate and compare human dermal fibroblasts isolated from neonatal (nHDF) and adult (aHDF) donors for their ability to promote *in-vitro* mineral deposition. In particular, cells were cultured in standard and in calcified medium and, at different time points, the amount of hydroxyapatite deposition and the expression/activity of proteins related to phosphate homeostasis as progressive ankylosis protein homolog (ANKH), ectonucleotide pyrophosphatase/ phosphodiesterase 1 (ENPP1) and tissue non specific alkaline phosphatase (TNAP) were measured.

Results indicate that neonatal fibroblasts are less prone to induce mineral deposition that adult cells, as demonstrated by the negligible occurrence of mineralized areas on the cellular monolayer. Moreover, the lower expression of ANKH and ENPP1 associated with a dramatic increase of TNAP activity in aHDF, compared to nHDF, may exert a key role in altering the PPi/Pi balance with age. These differences, being dependent on donors' age, are likely due to epigenetic changes (environmental factors and lifestyle) and may contribute to the reduced capability of aging fibroblasts to inhibit/counteract aberrant calcification.

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THE COLLAGGRECAN PROJECT: SYNTHESIS AND VISUALIZATION OF AN ARTIFICIAL PROTEOGLYCAN

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Glycoconjugates are highly critical components of the extracellular matrix. In particular the large PGs (aggrecan, versican etc.), which are mostly found in cartilages and in the joints of the locomotor apparatus, raise a growing interest because of the high morbidity of arthritic phenomena in an aging population. Although natural aggrecan and hyaluronic acid can be easily obtained in industrial amounts, the synthesis of engineered, custom-made molecules with different glycosaminoglycan side chains and different functional behavior seems promising. The many parameters involved (e.g., glycosaminoglycan species, sulfation type and pattern, molecular weight, number of side chains etc.) make possible to conceive an almost endless variety of aggrecan-like molecules, precisely tailored to specific functional demands.

In the present study chondroitin-6-sulfate chains were covalently bound to the lysyl and hydroxylysyl residues of a collagen molecule core (type I, from bovine) by reduction of an intermediate Schiff base. While in natural proteoglycans the side chains are always *O*-linked to their protein core, here the chains were *N*-linked in a way more reminiscent of natural cross-links. The reaction was confirmed by FTIR analysis.

For AFM imaging the material was resuspended in ultrapure water at 100 µg/ml and deposited onto freshly-cleaved mica pre-treated for 30 min. with 1M $MgCl_2$. Observations were carried out in Tapping Mode AFM in air with Nanosensors TESP-SS probes ($f \approx 300$ KHz, $k \approx 42$ Nm^{-1}). The molecules were readily visible, although structural details were recognizable with some effort because of the detrimental effect of the finite radius of the tip, and each collagen molecule appeared to be bound to 20 to 30 side chains. Biological tests are now underway.

A second approach involved the preparation of a collagen film by solvent casting from a 2 mg/ml collagen solution in 0.5M acetic acid; the film was then neoglycosylated as above with chondroitin-6-sulfate, washed twice in ultrapure water and ethanol and allowed to dry. The specimens showed a network of thick, banded collagen fibrils dispersed into a felt-like matrix of tangled collagen molecules; the surface was entirely covered with thin, slender chondroitin sulfate chains. The research is still in progress, but some of these specimens have already proved their biocompatibility and their ability to direct stem cells to differentiate in a specific way.

This work has been supported by Fondazione Cariplo, grant n. 2011-0270.

ADIPOSE TISSUE AS A NOVEL RESOURCE FOR BONE REGENERATION: ANALYSIS OF OSTEOGENIC POTENTIAL OF ADIPOSE-DERIVED MESENCHYMAL CELLS

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The novelty in mesenchymal stem cell research has been represented by the adipose tissue as a promising source of mesodermal derived-multipotent cells, called adipose stromal cells (ASCs). Adipose tissue can be harvested with a minimally invasive liposuction, and ASCs could overcome important constraints such as harvest site pain, morbidity and risks of infection. The aim of this study was to analyze the influence of different human adipose harvesting sites on ASC yield, proliferation, stemness, characterization and osteogenic potential when cultured in differentiating condition.

Eighteen specimens were collected by liposuction from 14 subjects (12 females and 2 males) with different age, body weight, height and body mass index: 6 samples were obtained from abdominal area, 5 from trochanteric area and 7 from breast.

Surface characterization showed the typical mesenchymal CD pattern (CD44, CD73, CD90, CD105) and negative expression of endothelial and hematopoietic markers (CD31 and CD45). No significant differences among the harvesting sites were found for ASC characterization, yield and stemness, collagen type I gene activation, alkaline phosphatase synthesis and mineralized nodules formation. Abdomen derived ASCs showed lower proliferation values than breast and trochanteric ASCs and higher values of RUNX2 and TGF 1. Finally, a significant synthesis of VEGF (vascular endothelial growth factor, important for its angiogenic role) was found in ASC cultures.

These preliminary results demonstrate that all sample of ASCs, regardless to harvesting sites and patient characteristics, are able to differentiate into osteoblasts. Mesenchymal cells adipose-derived could be a viable and abundant cell source useful in orthopedic regenerative medicine.

PHENOTYPIC PROFILING OF OSTEOTROPIC BREAST CANCER CELLS

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One of the preferred locations of metastases from breast cancer is the bone tissue. On the other hand, it should be recalled that mammary tumors with equal clinical diagnosis have a different course, and also different metastatic progression. Therefore, it would be helpful to have appropriate markers of osteotropism to test on the surgical cancer tissues, in order to predict the possible propensity of the breast cancer to generate bone metastases and to adequate the therapeutic plan.

We previously reported[1,2] on the setting-up of an in vitro model for the study of the osteotropic propensity of breast cancer cells and the influences exerted by the bone microenvironment on the cancer cells phenotype.

Viable bone fragments, deriving from surgery on traumatic lesions of young subjects were washed from bio-contaminants under sterile conditions and placed into cell culture capsules with the proper culture medium supplemented with foetal bovine serum, L-glutamine, L-ascorbic acid and antibiotics. The explants were kept for controlled timing in the humidified incubator in order to promote the release of resident cells, and then co-cultured with under-confluent breast cancer cells (SKBR3), until confluence. The bone fragments were then recovered, washed, placed again in culture dishes and monitored daily. The cells released from the bone fragments were then collected and processed for immunological characterization and proteomic profiling. The proteomic profiles of the cells seeded into the bone fragments were compared with the original cell culture, revealing an interesting differential proteomic pattern. The collection of identified proteins on the maps has reached up today the number of 373. Differentially expressed proteins between bone-seeded cells and wild type cells were about 30%. Among the differentially expressed proteins were several proteins belonging to the cytoskeleton remodelling and proteins of the class of calcium-binding cluster. The relevance of these protein clusters is discussed.

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POSTER SESSION

THE GENETIC COMPLEXITY OF PSEUDOXANTHOMA ELASTICUM (PXE) ACCOUNTS FOR PATIENTS' PHENOTYPIC HETEROGENEITY

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Pseudoxanthoma elasticum (PXE) is a rare autosomal recessive disorder characterized by progressive mineralization of elastic fibers particularly affecting skin, eyes and the vascular system. To date, more than 300 mutations in the ABCC6 gene have been discovered to cause PXE. Surprisingly, huge inter-individual phenotypic variability can be observed, even among family members bearing the same mutation, thus suggesting the involvement of modifiers genes. Moreover, it is generally accepted that in only 75%-80% of patients clinically diagnosed with PXE it is possible to demonstrate the presence of two causative ABCC6 mutations. Although technical issues cannot be ruled out, as well as the possibility that a number of mutations may take place in deep intronic regions, that, according to standard protocols, are rarely analysed, mutations and/or polymorphisms in other genes, in addition to those in ABCC6, may significantly contribute to the heterogeneity of the clinical phenotype. Therefore, we have started to perform a complete sequencing of ENPP1, GGCX and VKOR genes (i.e genes that have been already associated with pathologic mineralization) in 10 PXE patients diagnosed on the basis of typical clinical and histologic findings, but exhibiting ABCC6 mutation in only one allele. Numerous polymorphisms have been detected in all these genes, with the exception of the small VKOR gene. It has been already suggested that some polymorphisms, in a normal genetic setting, may affect clinical manifestations. At present, it is not known the effect of these polymorphisms in the presence of one causative mutation, and if a moderate pathologic phenotype might be developed, thus worsening, for instance, age-related complications. Interestingly, few patients exhibited one ABCC6 and one ENPP1 or GGCX causative mutation. In the light of these data, it could be suggested that monoallelic mutations in two different genes may synergistically act leading to a PXE or a PXE-like phenotype. Although a higher number of patients has to be screened and results must by evaluated by powerful statistical analyses in order to validate data and to establish possible correlations, these preliminary observations indicate that, in PXE, it is important to investigate more than one gene both for diagnostic and possibly prognostic significance, in agreement with the importance of gene networking in apparently monogenic inherited diseases as in PXE.

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A FUNCTIONAL PROTEOMIC OVERVIEW ON OSTEOGENESIS IMPERFECTA IN A MURINE MODEL OF THE DISEASE

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Osteogenesis imperfecta (OI) is an inherited disorder of the connective tissue which is mainly characterized by imperfectly formed brittle bone. In patients affected by the dominant form, the disorder is caused by mutations in one of the two genes encoding collagen type 1. In these subjects severity varies widely, spanning from perinatal lethality to very mild forms, also in the presence of an identical molecular defect. The OI murine model Brtl$^{+/-}$ exactly resembles such phenotypic heterogeneity showing either a moderately severe or a lethal outcome associated with the same Gly349Cys substitution in the 1 chain of type I collagen. In order to improve the comprehension of molecular basis of OI phenotypic variability and its extraskeletal systemic manifestations, we performed microarray and functional proteomic investigations in calvarial bone, skin and lung specimens from both mutant lethal and mutant alive Brtl$^{+/-}$ mice.
According to pathway analysis, Western blot and immunohistochemistry we predicted and proved that OI biomolecular aberrances are not only based on abnormal extracellular matrix deposition but also on an altered cytoskeletal organization as well on a general affection of extracellular/intracellular signaling and on cellular machinery malfunction[1,2]. While the mutant alive Brtl$^{+/-}$ mouse shows a protein expression pattern and cellular properties more similar to the WT, the mutant lethal animal presents consistent altered expression of proteins active in modulating cytoskeleton dynamics (*i.e.* vimentin, cofilin-1 and stathmin), signal transduction (*i.e.* TGF- & SMAD2/3, and focal adhesion distribution), and protein and cellular fate (*e.g.* proteasome subunits, alphaB crystalline and endoplasmin, maspin and Oct3/4). Worthy of note and in line with the recent tendency to consider OI a systemic disorder, the aberrances we observed in bone tissue from lethal mutants have been similarly detected also in their skin and lung biopsies.
In conclusion, we have pointed out some OI biomarkers with high potential for novel OI treatment attempts. In particular, we have described cytoskeleton as a key modulator of OI phenotype, as we at least in part proved in human[2].

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PAR6 INTERACTORS IN NORMAL AND TUMORAL CELLS

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The polarized phenotype is a hallmark of the normal epithelial tissues. Among the cellular pathways which participate to its biogenesis and maintenance, the PAR-aPKC system plays a pivotal role. The Par6 protein is involved in the connection of the junctional systems with cytoskeleton and associated proteins, so acting as a scaffold for the interaction between the GTPase CDC42/Rac1 and the aPKC[1] protein.
In the neoplastic cells the machinery of the cellular polarity is highly compromised, and the loss of polarity is a fundamental step for the acquisition of the malignant phenotype.
Our group has previously reported the different subcellular localization of the Par6 proteins in HB2 normal epithelial cells (cell membrane) and in 8701-BC breast cancer cells (cytoplasm)[2]. Moreover, the proteomic approach and MALDI-TOF mass

spectrometry allowed to identification of a set of Par6-interacting proteins apparently not correlated with the polarity[2].
The present study aimed to improve the knowledge about the role of the Par6 protein in the tumoral progression, by expanding the proteomic data and integrating them with the bioinformatic platforms available online (STRING, DAVID).
The results of this approach suggested a diversified role played by Par6 in the normal tissues with respect to the tumoral counterparts. In fact, it was observed that the major partners of Par6 in the normal cells were proteins involved in cytoskeletal stabilization, cell adhesion and cell differentiation, as expected from literature. Conversely the partners of Par6 detected in the cancer cells belong to the classes of cytoskeleton rearrangement for the filopodia protrusion, cell motility, cell proliferation and vesiculation, which are known to be be involved in many aspects of the cancer progression.
The work was co-funded by the Italian 5x1000 to COBS.
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LOW-INTENSITY PULSED ULTRASOUND EFFECTS ON MESENCHYMAL STEM CELLS ALONE AND IN COMBINATION WITH CERAMIC BIOMATERIAL

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Bone and articular cartilage lesions are hard-to-treat due to cartilage limited self-regeneration ability and process complexity. Tissue engineering provides alternatives to overcome surgery limitations. Bone growth and repair are under the control of biochemical and mechanical signals so in recent years have been evaluated several approaches to improve bone regeneration: osteoinductive biomaterials, stem cells, specific growth factors and biophysical stimuli. In particular low-intensity pulsed ultrasound (LIPUS) stimulation at 30 mW/cm^2 is an established, widely used and FDA approved intervention for accelerating bone healing in fractures and non-unions. The differentiation process of MSCs towards pre-osteoblasts to osteoblasts involves some changes in cell proliferation and activation or silencing of specific pathways leading to extracellular matrix synthesis, deposition and mineralization.
The aim of the study was to evaluate the effects of LIPUS alone and in combination with osteoinductive medium or ceramic biomaterial on human mesenchymal stem cells (hMSC) differentiation.
LIPUS with spatial averaged and temporal averaged (SATA) intensity at 30 mW/cm^2 was applied to hMSCs cultured in basal medium or osteogenic medium or into ceramic scaffolds at different experimental times. The analysis was focused (a) on the osteogenic specific pathways activation by gene expression analysis (early genes: *ALPL*, *COL1A1*, later genes: *RUNX2*, *BGLAP, MAPK6*) and related protein release (COL1a1, OPN, OC); and (b) on the maintenance of a little quantity of pluripotent hMSCs (CD73+/CD90+: 6%).
LIPUS treatment alone induce osteogenic differentiation in human MSCs without reducing completely their stemness, and is able to enhance the osteoinductive effects of ceramic scaffolds.

FUNCTIONAL PROTEOMIC STUDY OF FIBROBLAST CELL LINES FROM PATIENTS AFFECTED BY DIFFERENT RECESSIVE FORMS OF OSTEOGENESIS IMPERFECTA

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Osteogenesis imperfecta (OI) is a heritable connective tissue disease mainly affecting bone. Most of OI cases are caused by autosomal dominant mutations in one of the two genes encoding type I collagen. Rare recessive forms are instead due to deficiency of proteins involved in the overall metabolism of collagen I[1]. Mutations in *CRTAP*, *LEPRE1* and *PPIB* genes are causative of recessive types: VII, VIII, and IX, respectively. These genes code for proteins that form an endoplasmic reticulum complex involved in proline hydroxylation of collagen type I. Although OI is mainly a bone disorder, a skin outcome was also proved[2], and given the difficulty to obtain multiple bone biopsies from patients, the fibroblasts seem a proper alternative to study this disease.
In order to define molecular pathways affected by different types of recessive OI, we applied a functional proteomic approach in primary fibroblasts from recessive OI patients carrying mutations in *CRTAP*, *LEPRE1* and *PPIB* genes.
According to 2D-gel analysis, several significant differentially expressed proteins were detected among the three tested conditions and control fibroblast cell lines. Moreover, despite *CRTAP*, *LEPRE1* and *PPIB* genes encoded proteins are involved in different steps of the same biochemical process, some protein differences were also detected among investigated pathological conditions. Mass spectrometry was then applied and identified proteins were functionally processed by pathway analysis. Generated net suggested that cytoskeleton and nuclear organization are affected in recessive OI, as we recently proved in the dominant form[3]. Despite differentially expressed proteins implicated in these processes differ in recessive and dominant OI, we attempted to find similar affected cellular dynamics in OI forms independently of affected proteins. To this aim, we co-processed proteins changing in expression detected in fibroblasts from recessive OI patients with proteins belonging to or active in cytoskeletal organization that we described altered in dominant OI[3]. All these latter entered the net and vimentin and stathmin became central hubs. Based on literature and network data, the most interesting proteins were investigated also by Western blot.
In conclusion, our functional study has highlighted potential therapeutic targets for recessive OI.
The work was supported by Fondazione Cariplo [grant n. 2013-0612], Telethon [grant n. GGP13098].

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A BIOINFORMATIC APPROACH TO STUDY EARLY EVENTS IN ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is the most common form of dementia and the AD patients gradually lose cognitive function, control over their sense of orientation, their emotions, and other aspects of behaviour. Pathohistologically, AD is characterised by the presence of extracellular deposits of beta amyloid protein (A) in diffuse and neuritic plaques and intracellular deposition of hyperphosphorylated tau protein in neurofibrillary tangles[1]. Accumulation of A 42 is believed to be the earliest pathohistological feature of AD[2]. However, the early events in the onset of the pathology remain to be fully elucidated. As such, a greater understanding of the immediate and direct effect of the accumulation of this protein on neuronal cells may shed light on the mechanisms involved in the early, preclinical stage of the disease. To simulate early event in AD a cell model system in which LAN5 neuroblastoma cell were incubated for short time with a recombinant form of A 42 was utilized for a study of the proteome by mass spectrometry. Furthermore, a bioinformatic analysis, by using KEGG tool, indicated that some proteins were up or down regulated and the involvement of four pathway was identified. In particular we found down regulation of the spliceosome pathway. To confirm the existence of a suppressive effect of A 42 on the spliceosomal pathway, SmB/B′/N (a component of the spliceosomal machinery) was quantified. Proteins were extracted from LAN5 cells treated with A 42 at different concentrations and times, and a Western blot was performed. SmB/B′/N levels were found to decrease in a time and dose dependent manner relative to the control suggesting that impaired splicing can occur. However, further studies are necessary to fully elucidate the down-regulation effect of the spliceosome proteins in AD, and how this may contribute to the early event in this disorder.

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METABOLIC CONTROL OF HYALURONAN SYNTHASES

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Hyaluronan (HA) represents a perfect environment for cell migration and proliferation as we described for human aortic smooth muscle cells (SMC)[1,2]. Smooth muscle cells (SMC) in the presence of different stimuli, as inflammation, oxLDL, mechanical stress, produce large amount of HA as demonstrated in vivo in areas of atherosclerotic lesions. It has recently become evident that HA is an active modulator of proliferation and inflammation of the atherosclerotic plaque and in other inflammatory conditions as cancer microenvironment[3-6]. The control of the HA synthesis is critical in ECM assembly and cells biology. HA is produced on the plasma membrane by HA synthases (HAS1-3), which use cytoplasmic UDPGlcUA acid and UDPGlcNAc as substrates and UDP-sugar availability as well as the energy level of the cells are critical for the HA synthesis. The cell energy sensor AMP activated protein kinase (AMPK) leads to HAS2 T110 phosphorylation, which specifically inhibits HA secretion[7,8]. However, the most general sensor of cellular nutritional status is the UDPGlcNAc, which induces the intracellular protein glycosylation (O-GlcNAcylation). O-GlcNAcylation of HAS2 is present on serine 221 residue that induces a dramatic stabilization of the enzyme in the membranes increasing HA production[9]. Eventually we found a long non-coding RNA (NAT) for HAS2, which plays a role in this context involving p65 and NFkB pathway as ChIP analysis[10,11]. Unpublished data revealed that oxLDL are able to induce an increase of NAT for HAS2 in smooth muscle cells as well as other epigenetic modifications induced by P300 activity.

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G26/24 EXTRACELLULAR MICROVESICLES CONTAIN BOTH H1° PROTEIN AND RNA

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Extracellular vesicles (EVs) are released into the extracellular space from both tumor and normal brain cells. By releasing EVs which contain FGF2 and VEGF[1,2], astrocytes and neurons, co-cultured with brain capillary endothelial cells, are for example able to induce them to form a blood-brain barrier-like monolayer. On the other hand, membrane microvesicles (MVs) shed from G26/24 oligodendro-glioma cells, when added to primary cultures of rat cortical neurons, induce neuronal damage; the damaging effects include a strong reduction of neurite outgrowth, and apoptosis in about 75% of the cells[3]. The same amount of shed MVs induce apoptosis in about 40% of astrocytes[4]. These effects are probably due to Fas Ligand and TRAIL, two proteins, present in G26/24 vesicles, with well-known cell death inducing ability[5,6]. EV-mediated horizontal transfer of labeled proteins from oligodendroglioma cells to astrocytes in culture was also noticed[4].

We found that, in cultured astrocytes, as previously found in developing rat brain, the amount of the H1° linker histone increases during differentiation, while the level of its mRNA decreases, suggesting that its expression is mainly regulated at the post-transcriptional level[7]. On the other hand, G26/24 maintain high levels of both H1° protein and mRNA.

We recently found that these tumor cells release both H1° protein and mRNA, through EVs, into the culture medium[8]. We suggest that G26/24 oligodendroglioma cells, and possibly other tumor cells, can escape differentiation cues, and continue to proliferate by eliminating proteins, such as the H1° linker histone (and its mRNA) into the extracellular space.

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