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INVITED LECTURE

INVESTIGATING AND CHARACTERISING TENDON MICRO-MECHANICS

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Tendon plays a fundamental role in locomotion, facilitating energy efficient movement. However, not all tendons perform the same function, and the roles of our different tendons vary significantly. Energy storing tendons such as the Achilles must be highly extensible and elastic, to help us move efficiently, and to provide energy to help with locomotion. Such a function is not necessary in positional tendons, such as the digital extensor tendons of the hand. Here, accurate and careful positioning of the fingers is required, with a tendon that provides some dampening and can modulate muscle contraction[1]. All tendons are composed of the same hierarchical collagen arrangement, so to meet these disparate functional requirements, structural and compositional optimisation is required. Research in our group has focused on characterising the mechanisms by which different types of tendon function to transfer load, looking for mechanistic differences between tendons. Our data indicate that tendon extension and recoil relies on a series of hierarchical extension mechanisms all working together, with sliding between adjacent collagen units and rotation of helically arranged fascicles. However, we have evidence that different types of tendons utilise these mechanisms differently. More highly loaded energy storing tendons showing less sliding between structural units and behave more like springs. By contrast, positional tendons, which are more viscoelastic in nature, relying more heavily on sliding between fibres and fibrils for extension[2]. With a prevalence of tendinopathy in energy storing tendons, we are now interested to establish if the tendency towards damage is associated with the degree of specialisation. We are also looking to understand the cell environment within these different tendons, to see if mechanotransduction cues differ. We have recently developed a novel fibre composite material for tendon mechanobiology research, able to apply specific and controllable levels of shear and tension to tenocytes. Our preliminary data indicates that a shear environment modulates the mechanotransduction response of the cells, potentially providing us with insights into how cells may control the homeostasis of different types of tendon[3].

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ABSTRACTS

THE UNCOMFORTABLE LEAVINGS OF THE DECELLULARIZATION PROCESS

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Tissue engineered heart valves (TEHV) hold promise of ideal heart valve bioprostheses, avoiding inflammatory and immunological response, thromboembolism *phenomena* while capable of growth and repair. Xenogeneic biological matrices, as porcine heart valves, are considered the best choice for TEHV production considering both the anatomical structure and the presence of several trophic signals within the extracellular matrix (ECM). Unfortunately, not all the decellularization procedures (DP) used in TEHV production appear to be reliable and effective. The incomplete removal of both the xenogeneic Gal epitope (responsible for the hyperacute rejection) and/or the cytotoxic residues of detergents (hindering the cells penetrability within the matrix) is possibly related to the unsatisfactory clinical outcomes already experienced. We have determined the amount of residual detergent and carried out the evaluation of biocompatibility (by quantification of Gal leavings) in tissues decellularized according to established DPs. These DPs assumed to deliver promising results in literature, are focused on the action of detergents such as 1% deoxycholate (DOC)[1], 0.5% DOC/0.5% sodium dodecyl sulphate (DOC-SDS)[2] and 1% Triton X-100/0.4% cholate (TRICOL)[3]. As related to the removal of the Gal epitope only TRICOL ensures complete biocompatibility. In fact. 1% DOC leaves 43.3±1.2% of total antigen exposed, like the DOC-SDS procedure (49.1±0.8%, $p>0.05$). On the other hand, in 1% DOC DP 8.2±0.7% of the final acellularized tissue wet-weight was constituted by deoxycholate. The low amount of residual detergent in case of DOC-SDS and TRICOL (0.31±0.02% and 0.11±0.04% respectively) was comparable even if the difference was significant ($p=0.008$). Removal of such detergents is critical as unconjugated bile acids, like DOC and cholate, have been shown to accelerate elastolysis and to support the precipitation of insoluble calcium salts in a physiological environment. In turn, due to immunological activation, the presence of Gal is likely to sustain a constant inflammatory state in the human organism too. For these reasons we believe that the monitoring of such parameters should become a routinely tool test in the development of the future THEVs.

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MECHANICAL CHARACTERIZATION OF DECELLULARIZED BOVINE PERICARDIUM FOR TRANSCATHETER HEART VALVES PRODUCTION

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Transcatheter Aortic Valve Implantation (TAVI) is an inno-

vative emerging procedure consisting in the transcatheter implantation of expandable stent-based biological valves inside the native diseased valve through a trans-apical or trans-femoral approach. This procedure represents a less invasive alternative to classical valve transplantation thus avoiding sternotomy, extracorporeal circulation and cardiac arrest. Transcatheter Heart Valves (THVs) are currently made of bovine or porcine pericardium treated with glutaraldehyde (GA) that renders the graft non-permissive to cell repopulation and self-renewal, thus precluding a wider clinical use of these devices especially for children and young patients. A promising alternative to GA treatment is represented by decellularized bovine pericardial tissue. This work aims at the biomechanical characterization of native and decellularized bovine pericardium. Stress-strain relationships were collected by uniaxial tensile tests on samples from fresh and decellularized pericardium obtained by TRICOL (Triton X-100 and Sodium Cholate) and the brand-new TRITDOC (Triton X-100 and Sodium Taurodeoxycholate) procedures both allowing the production of acellular non-immunogenic xenogenic scaffolds devoid of the alpha-Gal epitope and retaining structural and morphological features of the native tissue[1-3]. According to different textural features previously determined two areas were identified and analyzed, corresponding to the anterior left ventricle (ALV) and the posterior right ventricle (RPV). The ALV region exhibits higher mechanical stability than the RPV, both in native and decellularized samples: 0,413±0,024 *vs* 0,281±0,024 for native samples; 0,518±0,022 *vs* 0,358±0,033 for TRICOL samples; 0,456±0,026 *vs* 0,364±0,026 for TRITDOC samples (mean MPa±standard error, $p<0,05$). Moreover, decellularized bovine samples demonstrate higher tensile strength than native pericardium. In conclusion, TRICOL and TRITDOC procedures allow effective cell removal and increase the mechanical stability of bovine pericardium. Indeed, decellularized bovine pericardial patches derived from the ALV region could be the ideal scaffold for engineered transcatheter heart valve manufacturing.

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FIBRONECTIN, LAMININ AND TYPE IV COLLAGEN ARE KEY COMPONENTS OF THE SINUSOIDS-ASSOCIATED MEGAKARYOCYTE "NICHE" WITHIN BONE MARROW

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Megakaryocytes (Mks) develop within the bone marrow environment by interacting with different extracellular matrices (ECMs) located at bone and vascular structures level. Recent evidences demonstrated that Mks contribute to the establishment of stem cell niches by regulating matrix deposition from environmental cells, releasing pro- or anti-angiogenic factors and ECM cross-linking enzymes, and supporting fibronectin fibrillogenesis. In this work we have analyzed the spatial distribution of Mks and ECMs by immunofluorescence in murine femur sections. We found that Mks were predominantly located in the femur diaphysis with only 20% of Mks within 50 m from the endosteal surface and more than 80% of Mks located less than 50 m from a sinusoid. Correlation between Mk distance from sinusoids and dimension suggested a gradient of maturing Mks towards the vascular niche. Next, we deciphered bone marrow ECM composition by western blotting and mapped the location *in situ* of different collagens (I, III, IV, VI) and glycoproteins (fibronectin, thrombospondin, laminin, von Willebrand factor). We found that all these proteins were differently located in the endosteal, arteriolar and sinusoidal districts supporting the concept that regulation of hemopoiesis, in the bone marrow, may also depend from matrix distribution. Further, we showed, for the first time, that Mks were surrounded by a pericellular matrix mainly composed of fibronectin, laminin and type IV collagen. Interestingly, these three proteins were also demonstrated to promote thrombopoietin-dependent Mk differentiation in *in vitro* cultures of bone marrow hemopoietic progenitor cells (3,53±0,86, 1,19±0,11, 1,40±0,23 fold increase, respectively). Finally, fibronectin, laminin and type IV collagen were also demonstrated to be expressed and synthesized by differentiated Mks *in vitro* as demonstrated by PCR and western blotting analysis. All together these results suggested that Mks are important ECM-producing bone marrow cells and that released ECMs support megakaryopoyesis and concur to the generation of bone marrow niches.

BETA-ARRESTIN 1 IS INVOLVED IN THE INFLAMMATORY RESPONSE STIMULATED BY HYALURONAN OLIGOSACCHARIDES IN MOUSE CHONDROCYTES

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Beta-arrestin 1 is an adaptor protein that leads termination of G protein activation and seems to be involved in the mediation of inflammatory response. Interleukin-1beta IL-1beta elicits the expression of inflammatory mediators through a mechanism involving the Hyaluronan (HA) depolymerization that contributes to toll-like receptor 4 (TLR-4) and CD44 activation. Stimulation of both receptors induces the nuclear factor kappaB (NF-kB) activation that in turn, through the canonical pathway involving the transforming growth factor activated kinase-1 (TAK-1), stimulates the transcription of pro-inflammatory mediators responsible of the inflammation response. We aimed to investigate the involvement of beta-arrestin 1 in an *in vitro* model of IL-1beta-induced inflammatory response in mouse chondrocytes. Chondrocyte treatment with IL-1beta produced a significant increase in TLR-4, CD44, beta-arrestin 1, TAK-1, and serine/threonine kinase (Akt) mRNA expression and of the related protein levels. The NF-kB was also markedly activated with consequent tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS) upregulation. Treatment of IL-1beta-stimulated chondrocytes with beta-arrestin 1 or/and Akt or/and TAK-1 specific inhibitors significantly reduced all the parameters, although the inhibitory effect exerted by the canonical pathway mediated by TAK-1 was more effective than that of beta-arrestin 1. The activation of NF-kB induced by beta-arrestin-1 was mediated by the Akt pathway as showed by chondrocytes stimulated with IL-1beta and treated with a specific Akt inhibitor. Finally a specific HA blocking peptide (Pep-1) confirmed the inflammatory role of degraded HA as mediator of IL-1beta.

HYALURONAN SYNTHESIS IS REGULATED BY INTRACELLULAR O-GLCNACYLATION OF HAS 2

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Large body of evidence supports the idea that microenvironment plays a critical role in several pathologies including atherosclerosis and cancer. The amount of hyaluronan (HA) often reflects the progression of the diseases as it promotes neo-angiogenesis, cell migration and inflammation and its synthesis is regulated by several factors as the phosphorylation of synthetic enzyme HAS2[1] as well as specific drugs reducing the UDP precursors[2]. In this work, based on human smooth muscle cell model, we studied the intracellular regulation of HA synthesis at molecular level. The hexosamine biosynthetic pathway (HBP) may increase the concentration of HA precursor UDP-N-acetylglucosamine (UDP-GlcNac) leading to an increase of HA synthesis[1]. In this study we addressed the issue trying to shed light on HA synthesis regulation. The flux through the HBP in the regulation of HA biosynthesis in human primary aortic smooth muscle cells (AoSMCs) was studied, as UDP-GlcNac is the donor of GlcNac for O-GlcNacylation by which the GlcNac attaches to ser/thr residues via an O-linked glycosidic bond. We found that the inhibition of O-GlcNacylation strongly reduced HA production whereas treatments that induced protein O-GlcNacylation increased HA secretion. Gene expression studies done by quantitative RT-PCR revealed that Hyaluronan synthase 2 (HAS2) mRNA was the most sensible to O-GlcNacylation and accumulated after its induction. Although factors governing constitutive HAS 2 transcription are to be elucidated, we found that the transcription regulator YY1 activates HAS2 expression after O-GlcNacylation. Using IP and recombinant 6myc-HAS2 we demonstrate that HAS2 is O-GlcNacylated and indentify the residue involved. Finally, as cell migration and adhesiveness are critical factors for neointima formation and progression, we quantified AoSMCs motility and monocytes binding and found that O-GlcNacylation increased cell invasion and inflammatory cell recruitment.

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MOLECULAR MECHANISMS INVOLVED IN HYALURONAN SECRETION BY AORTIC SMOOTH MUSCLE CELLS AFTER OXIDIZED LDL EXPOSURE

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The crucial event in atherosclerosis onset is the retention of lipoproteins (LDL) within the arterial wall due to endothelial dysfunction/lesion. The LDL accumulation triggers a cascade of events, such as LDL oxidation and fusion, ultimately leading to foam cell formation and lipid deposition. Such process starts a local inflammatory response with hyaluronan (HA) accumulation in the *intima* of the wall, leading to the thickening of the vessel. A recent study highlights the emerging role of smooth muscle cells (SMCs) in the early stages of atherosclerosis[1] because of their ability to migrate and proliferate in response to different stimuli. The aim of this study is therefore to shed light on the mechanisms involved in the incremented HA deposition. Using different types of human LDL (n-normal, ox-oxidized, agg-aggregated) we measured the HA production by aortic SMC (AoSMCs). oxLDL are more efficiently internalized by the AoSMCs as shown by fluorescence microscopy and influence the proliferation of cultured AoSMCs in a dose-dependent manner without affecting cell viability in a range from 10 to 50 ug (protein). Moreover, when added to the culture medium, oxLDL are able to increase the production of HA as shown by HPLC analysis and by exclusion assay, enhancing cell motility and monocyte adhesion. Considering that oxLDL are the most effective HA inducers we investigated the role of LDL scavenger receptors on HA synthesis, highlighting the importance of the LOX-1 receptor. Using a specific antiLOX-1antibody we were able to demonstrate that the LDL internalization is due to these uncontrolled receptors. As it was recently demonstrated that ER stress in cells may induce HA synthesis, we measured the expression of several ER stress markers: the results showed that the markers are very early expressed by the cells (24 h) and the HA production followed later (48h). Considering that ox-LDL drive high level of cholesterol, we depleted such particles of cholesterol obtaining a decrease of HA compared to control. Nevertheless, cholesterol alone did not increase HA levels.

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ROLE OF WATER IN TENDON BIOMECHANIC

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In tendon functional mechanical role is supported by the extracellular matrix whose macromolecules consist of collagen, proteoglycans, a small amount of glycoproteins and elastin, and water. Authors usually report that the insoluble fibrillar collagen, mainly consisting of type I collagen, represents the 80-90% of the dry weight. However water is the real major component of tendon because it represents the 55%-70% of the total wet weight. Fibrillar collagen arrays in a repeated, alternated and hierarchical handedness, from molecules to supra molecular structures, which increases the resistance of fibrils to tensional loads/elongation. In particular three polypeptide left-handed chains coil around each other to form a unique triple right handed tropocollagen helix. Five tropocollagen molecules stag side-by-side and twist in a left-handed helix forming a single microfibril. Microfibrils array into rightward helices to form a single fibril. Then fibrils form fibres running mostly parallel and densely packed with an almost straight course interrupted only by periodic crimps where fibrils always twist leftward. Interfibrillar spaces are filled by proteoglycans like decorin and a smaller amount of biglycan, whereas large proteoglycans like aggrecan and versican are supposed to be located in the inter fibre spaces. Collagen fibers probably retain the main part of interstitial bound water whereas proteoglycan aggregates could retain large amount of free water. The morphological and biomechanical behavior of collagen fibrils/fibres under physiological

stretching in Achilles tendon of rat were studied at the polarized light microscope, TEM and SEM. Under stretching, fibre crimps straightened and disappeared and fibril diameter reduced. We believe that an increased molecular packing with a fluid displacement occurs in fibril under tension. We suggest that both small (decorin and biglycan) and large proteoglycans (aggrecan and versican) binding large amout of water could play a role in favouring and regulating a fluid shift or a water flow during stretching and recoil of tendon. This hydrodynamic mechanical model is supported by a mechanical role of muscle and tendon sheets during physical activity.

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PROLIDASE DEFICIENCY: FROM THE MOLECULAR BASES TO A FUNCTIONAL RESCUE IN FIBROBLASTS FROM PD PATIENTS

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Prolidase deficiency (PD) patients have a reduced prolidase activity leading to a shortened life expectancy. Even thought some causative mutations were identified, no information about the causes of the enzyme inactivity and no therapies are available. In order to further investigate PD pathophysiology, we selected three mutations affecting different regions of the protein and representing the most frequent defects in PD patients: 231delY, E412K and G448R. The recombinant mutant enzymes produced in *E. coli* showed very low catalytic efficiency. A compromise ability to bind the cofactor was found in hRecProl231delY. A thermal instability and a delay in the dimerization process were detected in all mutant forms as well as changes in their structure, that was also confirmed by *in silico* analysis. Thanks to the deep biochemical investigation of the three causative alleles, we identified in protein perturbed folding/instability, and in the consequent partial protein degradation, the main cause for enzyme inactivity. The demonstration of folding impairment led us to attempt to stabilize the mutant proteins in patients fibroblasts through the induction of Heath Shock Proteins (HSPs) expression. An *in vitro* HSPs stimulation was performed by means of cells incubation at 48°C followed by the analysis of prolidase activity and protein expression level. Although the prolidase activity was low in all the mutants with respect to the control, and a partial loss of the protein was caused by the heating procedure, following 15 min of heat shock in the G448R and in the E412K lysates the prolidase activity was increased. These data suggested that high temperature led to easier degradation of the poorly structured mutant prolidase, while improved stability of newly synthesized mutant molecules was found when higher levels of chaperones were present. These findings suggested that drugs effecting the intracellular chaperon environment could be tested for PD treatment. Further investigation are needed to evaluate such approach *in vivo,* starting from a proper murine model.

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CANT1 AFFECTS PROTEOGLYCAN SYNTHESIS IN FIBROBLASTS FROM DESBUQUOIS DYSPLASIA PATIENTS

M. Luca[1], F. De Leonardis[1], R. Tenni[1], G. Cetta[1], A. Forlino[1], C. Huber[2], M. Bertoli[2], M. Fradin[2], M. Le Merrer[2], C. Le Goff[2], V. Cormier-Daire[2], A. Rossi[1]
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Desbuquois dysplasia (DD) is a severe autosomal recessive chondrodysplasia characterized by antenatal and postnatal growth retardation, short stature, multiple dislocations, and advanced carpal ossification. Two forms have been distinguished on the basis of the presence (type 1) or the absence (type 2) of characteristic hand anomalies. Studying DD type 1 families, several mutations in the *Calcium-Activated Nucleotidase 1* gene (*CANT1*) have been identified[1,2]. CANT1, a member of the apyrase family, is a calcium activated nucleotidase of 401 amino acids that preferentially hydrolyzes UDP followed by GDP, CDP and ADP. The substrate specificity as well as the cellular localization (ER and/or Golgi) suggest that CANT1 might be involved in glycoprotein synthesis. In addition since DD shares phenotypic features with other chondrodysplasias characterized by defects in cartilage proteoglycan sulfation, we hypothesized that CANT1 may play a role in proteoglycan synthesis. To test this hypothesis, fibroblasts from two DD patients homozygous for the p.R300H and p.P245RfsX3 mutations respectively, and four controls were double labelled with [^{35}S]sulfate and [^{3}H]glucosamine. In patients fibroblasts glycosaminoglycan (GAG) synthesis was within normal value under basal condition, however a significant reduction of GAG synthesis was observed in presence of β-D-xyloside, a compound which enhances synthesis of chondroitin and dermatan sulfate chains acting as a chain initiator. Furthermore, gel filtration chromatography on Superose 6 of GAGs released from newly synthesized proteoglycans after β-elimination demonstrated that GAG chains were shorter in the patients compared to the controls. Interestingly hyaluronic acid synthesis, which occurs in the plasma membrane, was within normal level in the patients cells, confirming the involvement of CANT1 in the ER/Golgi compartment. Overall these data suggest that CANT1 plays a role in proteoglycan metabolism and support its involvement in the endochondral ossification process.

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URINARY TRYPSIN INHIBITOR IS A MARKER OF RENAL DISEASE IN FABRY'S PATIENTS

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Fabry's disease is a rare X-linked lysosomal storage disorder caused by deficiency of -galactosidase A[1], that leads to accumulation of glycosphingolipids in many cell types and tissues. The disease manifestation usually starts in childhood and, in adulthood, the progression of the pathology may lead to renal and cardiac damages, and high propensity to develop cerebral ischemic stroke resulting in decreased life expectancy[2]. Early biochemical markers of Fabry's nephropathy, routinely used in clinical settings, are microalbuminuria or proteinuria. These markers are useful for documenting the occurrence of renal impairment, at an early stage, and indicate the need to introduce the appropriate enzymatic replacement therapy before renal damage becomes irreversible. Urinary trypsin inhibitor (UTI) is a small chondroitin sulphate (CS)-proteoglycan whose levels are usually low in healthy individuals but are increased in several pathological conditions including renal diseases[3]. Since renal damage is one of the major complications of Fabry's disease, the aim of this research was to evaluate if the UTI excretion could represent a useful indicator of early stage renal disease in these patients. UTI levels were evaluated on both plasma and first-morning urine samples from Fabry's patients (n=22), sorted among patients with overt renal disease (RD patients, n=11) and patients that did not show any obvious renal impairment (NRD patients, n=11), and age-and sex-matched, healthy controls (n=43). Overall, urinary UTI levels were significantly different between patients and controls (+60%, p=0.005) due to higher UTI levels in RD Fabry's patients (+84%, p=0.0001). By subsorting RD group in patients with only proteinuria or renal damage, no differences were evidenced demonstrating that the increase of urinary UTI levels in Fabry's patients may be another biochemical marker of early stage renal disease in these patients. No differences were evidenced in plasma levels of CS isomers, highlighting a specific association between urinary UTI levels and renal impairment. These results suggest that urinary UTI level could represent a useful marker in monitoring renal functionality in Fabry's patients. Further studies are needed to find out if urinary UTI levels could be an earlier marker of renal damage compared with proteinuria.

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SULODEXIDE INHIBITS HEPARANASE-1 AND PREVENTS FGF-2-INDUCED RENAL EPITHELIAL-MESENCHYMAL TRANSITION

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Several chronic kidney diseases, especially diabetic nephropathy (DN) progress to kidney failure and a common hallmark of the development of tubular-interstitial fibrosis Epithelial-mesenchymal transition of tubular cells is a widely recognized mechanism that sustains interstitial fibrosis and is triggered by several factors such as FGF-2. The signaling of FGF-2, a growth factor involved in this mechanism, is regulated by glycosaminoglycans. We recently proved that Heparanase, an endoglycosidase that cleaves heparan sulfate[1], is implicated in the pathogenesis of diabetic nephropathy and is necessary to FGF-2 for the induction of tubular cells transition[2]. Since well-known Heparanase inhibitors are heparin(s) and sulodexide, a low-molecular weight heparin – dermatan sulphate blend, is effective in the treatment of DN[3] we investigated how Sulodexide could affect HPSE and its effects on tubular renal tubular cells. We have investigated the inhibition by sulodexide and its components of Heparanase-1 buy an ELISA assay. We and have analyzed its effect on the epithelial-mesenchymal transition of tubular cells by real time gene expression analysis, zymography and migration assay. Results show that sulodexide is an effective heparanase-1 inhibitor, exclusively in virtue to the heparin component, with an IC50 of 5 g/ml. In FGF-2 treated tubular cells, Sulodexide also prevents the over-expression of the mesenchymal markers SMA, vimentin and fibronectin and the motility increase, *i.e.* the epithelial-mesenchymal transition of tubular cells. Moreover, sulodexide prevents FGF-2 induced heparanase-1 and MMP9 increase switching off the autocrine loop that FGF-2 activates to support its signal. The findings highlight the capacity of sulodexide to inhibit heparanase-1 and to control tubular fibrosis triggered by epithelial-mesenchymal transition. In conclusion, these sulodexide activities support the value of this agent in controlling the progression of nephropathy to renal failure.

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INVITED LECTURE

AGING AND LIFESTYLE ON THE MUSCOLOSKELETAL SYSTEM: FOCUS ON BONE AND TENDON CELLS

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Bone is a connective mineralized tissue that is subject to a great variety of intrinsic and extrinsic influences, and in particular to ageing, systemic diseases as osteoporosis, drugs and lifestyles.The burden of ageing and osteoporosis is increasing in all societies, uncorrectlifestyles representa avoidable harmful risk factor that negatively affects healthand life expectancy. In particular, obesity, alcoholism and tobacco smoking are some of the most common causes of adverse effects also on bone. It is demonstrated that high intake of alcohol increases the risk ofosteoporosis and fractures. Various studies on alcohol-induced bone changes highlighted that bone remodeling is altered and new bone formation is impaired (decreased osteoblast activity, reduced serum osteocalcin, vitamin D deficiencyfrom malnutrition) while small changes occur in bone resorption. *In vitro* studies on osteoblasts demonstrate that alcohol exposure reduced cell viability, osteogenic capacity and bone formation, with a more significant effect than estrogenic deficiency. Regarding tendon tissue, little is known about tenocyte behaviour during aging and, especially, in presence of estrogen deficiency. The proliferation and metabolism of tenocytes isolated from the Achilles tendons of middle aged or ovariectomised rats compared to young rats was *in vitro* evaluated. An *in vitro* model of micro-wound healing was also used to assess age and estrogen deficiency related differences in tendon healing. Proliferation and tenocyte biosynthesis are negatively affected both by aging and estrogen deficiency, even though estrogen deficiency exerts a greater negative effect than aging in culture. In micro-wound healing model, estrogen deficiency showed a negative effects on tendon healing and lower cell proliferation,lower cell speed migration and altered synthetic activity were observed in comparison with young tissue derived cells.On the contrary a moderate running exercise positively influence the biological activity of tenocytes which particularly increase collagen type I synthesis, with the aim of enhancing the efficiency of loading transmission. Theseaspects should be further clarified with appropriate preclinical studies and have a potential role in bone-biomaterial interactions and tissue engineered therapies for both bone and tendon healing.

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ABSTRACTS

THE ROLE OF VITAMIN K-DEPENDENT PROTEINS IN THE PATHOGENESIS OF PSEUDOXANTHOMA ELASTICUM

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Pseudoxanthoma elasticum (PXE) is a genetic disease characterized by progressive mineralization of the elastic component, which has been related to a reduced expression of the active form of matrix gla protein (MGP), a strong inhibitor of ectopic calcifications. To be fully effective, MGP has to be carboxylated (c-MGP) through a vitamin K-dependent pathway. The observation that in PXE, patients have lower levels of circulating vitamin K, arose the question whether elastic fiber mineralization is due to an insufficient amount of the vitamin reaching peripheral connective tissues, or to the inability of PXE mesenchymal cells to utilize the vitamin. Unexpectedly, vitamin K supplementation does not exert beneficial effects on soft connective tissue mineralization in the PXE animal model. For a better understanding of the role of vitamin K-dependent carboxylation of MGP in PXE pathogenesis, aim of the present study was to investigate the effects of vitamin K1 (phylloquinone) and vitamin K2 (menaquinone-4, MK-4) supplementation on control and on PXE dermal fibroblasts cultured in standard conditions and in calcifying medium. Results demonstrate that vitamin K1 and K2 can be taken up and accumulated at similar levels by control and PXE fibroblasts, that the carboxylation process can be consequently similarly up-regulated, that both vitamins K1 and K2, independently from concentration, similarly down-regulate the expression of CALU in all cell strains, whereas changes are negligible in the case of PDI, indicating that the effect of vitamin K supplementation on the expression of ER-proteins involved in vitamin K cycle is not pathway-specific. Surprisingly, MGP cannot be adequately carboxylated, even at increased levels of vitamin K. It can be therefore excluded that PXE fibroblasts are not capable to utilize the vitamin, thus suggesting that altered MGP characteristics/properties could contribute to defective carboxylation. Moreover, the observation that in an *in vitro* calcification model, both vitamin K1 and K2 are ineffective in inhibiting the mineralization process, also in control fibroblasts, *i.e.* in cells that do not exhibit reduced cMGP, may underline the importance and the complexity of the extracellular environment in mineral deposit formation and in regulating cell behavior.

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ARIADNE'S THREAD. HOW TO EXPLORE THE LACUNAR-CANALICULAR SYSTEM OF BONE

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The osteocyte lacunar-canalicular system forms a highly complex network, and its morphological and functional aspects are still object of discussion. In particular are well

known the difficulties of visualizing and representing the canalicular labyrinth. Several studies exist, carried out either by serial sectioning of embedded specimens, or by confocal laser scanning microscopy, or with FIB/SEM imaging. With all these techniques, however, the persistence of the dense bone matrix hampers the visualization of the finer details of the canalicular network. In an attempt to obtain a better visualization we tried to improve the corrosion casting technique, applying its potential to the osteocyte lacunar-canalicular network. In the present research the methyl-metacrylate (MMA) casting technique was applied to human cortical bone in order to evidentiate the 3-D organization of the osteonic lacunar-canalicular system. The infiltration of the MMA monomer into the vascular canals, and hence into the lacunar-canalicular system, was driven by capillarity, helped by evaporation and the resulting negative pressure. The resin penetration was complete in the Haversian canal but limited to a depth of 4-5 layers of lacunae around the canal itself, suggesting that each secondary osteon is a closed system limited by an impenetrable cementing line. The casts duplicated the shape, position and connections of the lacunae, and allowed an unrestricted exploration of lacunar-canalicular network without manipulations such as cutting or sawing. Two systems of canalicula could be distinguished: the equatorial, which connects osteocytes lying on the same concentrical level, and the radial, which interconnects different levels. The equatorial canalicula radiate from the lacunar border on a planar surface around the lacuna, forming straight and basically parallel canals; the radial exhibit a predominant direction perpendicular to the equatorial plane. The architecture of the lacunar-canalicular network obtained by the MMA casts was then compared with theoretical models of the fluid circulation in the osteonic bone.

THE ABNORMAL ENDOCHONDRAL OSSIFICATION IN PERINATAL LETHAL OSTEOGENESIS IMPERFECTA (TYPE II)

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Three cases of type 2 osteogenesis imperfecta were diagnosed by ultrasounds examination respectively at the 15th, 21st and 22nd week of gestation. In each case termination of the pregnancy 1 month later allowed an x-rays complete skeletal survey and a histopathological study of the bones. Morphometry was completed by comparison with an aged-matched population without skeletal diseases. The cartilage model of the long bones and of the vertebrae was normal, with the regular condrocytes stacking, hypertrophy and calcification of the cartilage inter-columnar septa. No differentiation of the osteoblasts was observed on the surface of the calcified cartilage septa, therefore there was no formation of primary and secondary trabeculae inside the endochondral ossification center. A scanty bone matrix apposition and few lining osteoblasts were observed beneath the periosteum of O.I. fetuses. No fractures of the calcified cartilage scaffold occurred in the vertebral body ossification centers up to the 23rd week, but a large number of them were observed throughout the metaphysis and the diaphysis. An attempt of healing was activated, but it was characterized by the production of cartilage matrix with the early reported failure of osteoblasts differentiation and lack of bone matrix deposition. Multinuclear osteoclast-like cells (number/section and nuclei/cell) were significantly higher in O.I. than in controls ($p<0.001$), but they were not actively resorbing cells because did not form Howship lacunae and showed a remarkable condensation of the nuclear chromatin. Inhibition of osteogenesis as well as of modeling and remodeling appeared as the characterizing features of type 2 osteogenesis imperfecta.

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RESPONSIVITY OF AORTIC VALVE INTERSTITIAL CELLS IN CULTURES AT DIFFERENT NORMOPHOSPHATEMIC-LIKE CONDITIONS. CALCIFIC EVENTS VERSUS AUTOPHAGOCYTOSIS AND APOPTOSIS

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To mimick dystrophic calcification, occurring in normophosphatemic conditions, bovine aortic valve interstitial cell (bAVIC) cultures containing different Pi concentrations (0.8, 1.3, and 2.0mM) were accomplished, including borderline ones on respect to hypo- and hyperphosphatemic-like conditions. Neither cell death signs nor appearance of calcific nodules were observed except for bAVIC cultures containing 2.0mM Pi. At 3-day-long incubation, immunoreactivity to specific marker of mature autophagosomes MAP1-LC3A was higher for control bAVICs and decreased for cultures containing 0.8, 1.3, and 2.0mM Pi in the order. Moreover, there was a time-dependent decrease for all cases. On detecting apoptosis occurrence, low positivity resulted to caspase-8 and almost unreactivity to caspase-9, caspase-3, and annexin-V. For 0.8mM-Pi-cultures and, at greater extent, 1.3 ones, bAVICs showed autophagocytosis to have started and atypically progressed, with (i) a progressive RER enlargement causing cytoplasm compartimentalization into hollows/canalicular spaces, which confined altered organules, and (ii) parallel autophagosome loss. Conversely, most bAVICs in 2.0mM-Pi-cultures were affected by degenerative events, with appearance of phthalocyanin-positive material outcropping at cell surface and acting as hydroxyapatite nucleator, besides being source of real calcospherulae. Spectrophotometric estimations of calcium amounts and alkaline phosphatase activity were consistent with the morphological data, resulting (i) similar values between control cultures and those containing 0.8 and 1.3mM Pi and (ii) significantly higher values for 2.0mM Pi containing cultures *versus* those as at point (i). In conclusion, the immunohistochemical data suggest that (i) autophagocytosis might be an epiphenomenon simply related to cell survival mechanisms and (ii) apoptosis to be not significant in promoting the calcific process. The ultrastructural detection of abnormal autophagocytosis suggests a derangement of this process but without apparent correlation with calcification. Interestingly, the propensity of bAVICs to undergo procalcific degeneration resulted to correlate with Pi concentration in such a way that a differential discrimination of this parameter within the conventional normophosphatemic range seems to be mandatory for a proper evaluation of dystrophic valve calcification risk.

THE TUMOR MICROENVIRONMENT AS TARGET FOR CANCER THERAPY AND CHEMOPREVENTION: MATRIX METALLO-PROTEASES AND BEYOND

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The association between connective tissue diseases and malignancy has been object of debate. Members of the emerging family of regulatory proteins CCN* play various roles in angiogenesis and tumor growth, such as regulation of cell division, chemotaxis, apoptosis and motility. In this perspective, the tumor microenvironment, a "complex society" composed of many cell types and extracellular matrix components, plays a pivotal role in the regulation of angiogenesis and also in cancer progression. We aim to identify molecules and pathways to prevent tumor development by targeting the microenvironment and inflammatory angiogenesis involved in the carcinogenic process[1,2]. We have shown that different molecules, such as flavonoids, antioxidants and triterpenoids, operate in the tumor microenvironment inhibiting the recruitment and/or activation of endothelial cells and innate immune cells. The green tea flavonoid epigallocatechin-3-gallate (EGCG), and Alpha lipoic acid (ALA) prevent angiogenesis in the Matrigel sponge angiogenic assay *in vivo* and inhibit the growth of the highly angiogenic Kaposi's sarcoma tumor cells (KS-Imm). The flavonoids suppressed the IkB/NF-kB signaling pathway in the presence of NF-kB stimulation by TNF , and reduced expression of NF-kB target genes. The repression of this pathway suggests anti-inflammatory and anti-angiogenic effects of the anti-oxidant compounds. Among angiopreventive molecules, we tested also triterpenoids, hyperforin and isoflavon Xantohumol (Xn). Analyses of gene expression regulation by anti-angiogenic chemopreventive compounds in primary human umbilical endothelial cells (HUVEC) in culture through Affymetrix GeneChip arrays identified overlapping sets of genes regulated by these compounds. In addition, we are generating and testing novel formulations of Xn by using *in vitro* angiogenesis assays. Preliminary experiments confirm the enhanced anti-angiogenic efficacy of these modified derivatives. Finally, we are chemically modifying metalloproteinase inhibitors enhancing their activity against substrates deriving from endothelial and tumor cells. These data show that molecules interfering in angiogenic processes could be very useful in inhibiting microenvironment alterations that contribute to neoplastic transformation.

* *Connective tissue growth factor, Cystein rich protein and Nephroblastoma over-expressed gene.*

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IDENTIFICATION OF TYPE V COLLAGEN-BINDING PROTEINS IN 8701-BC BREAST CANCER CELLS

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The cancer progression is characterized by the ability of malignant cells to alter the cell-cell and cell-matrix crosstalk and to change the composition of extracellular matrix (ECM) in order to create a more favorable environment for cell dissemination. Among components of ECM, collagens are highly abundant and play a dual biological role by acting as scaffold proteins (conferring elasticity and plasticity to the matrix) and as regulators of cell motility and proliferation (via receptors-mediated signaling). The type V collagen, belonging to the fibrillar collagens, represents less than 1% of total collagens of physiological ECM, but its amount increases up to 10% in some cases of breast carcinomas (BC). As previously reported by our group[1], in opposition to other BC-associated collagens, such as OF/LB collagen, type V exerts a restrictive effect on ductal breast carcinoma derived 8701-BC cells proliferation. Moreover, the 8701-BC cells seeded on selected collagen substrata show differences in cell morphology, adhesion degree and growth rates. It was also immunologically recognized a 67kDa protein as a major receptor for collagen V[2,3]. We here report the spectrometric identification of other type V binding proteins which could act as co-receptor or modulators of cell-collagen V interaction. The 8701-BC cells were collected, lysed and subjected to cell fractionation. The membrane-derived protein fraction was subjected to affinity chromatography by incubation with a Sepharose 4B-CNBr resin coupled with collagen V. After removal of the unbound fraction, the bound proteins were collected and subjected to SDS-PAGE electrophoresis. The gels were stained by Coomassie Blue and each band was submitted to MALDI-TOF mass spectrometry. So far, we have identified 11 proteins, including isoforms, namely GRP78 (3 bands), PDIA3 (2), PDIA6 (2), Enolase A and Annexin A2 (3). Literature data reported that each of these proteins, besides their primary cytoplasmic location, may translocate to the cell surface where they may perform additional diversified functions. We believe that present result give new insight in the role of the identified proteins and improve the knowledge about the "actors" of the interaction between collagen V and neoplastic cells opening new scenarios on the effects of ECM in cancer progression.

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PROTEOMIC SIGNATURE OF BREAST CANCER TISSUES FOR PATIENTS STRATIFICATION

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Breast cancer is a complex and heterogeneous disease traditionally classified on the basis of morphological and molecular examination. Recent advances in biotechnologies, have provided further possibility to classified breast cancer into additional subtypes with different prognosis (*e.g.* luminal, basal-like, claudin-low, etc.), for which some gene signatures are already available. However, several investigations have elucidated the active role of the microenvironment (stromal cell and extracellular matrix molecules) on breast cancer progression. Our group has previously reported that permissive and restrictive signals are emanating from the tumor stroma[1,2], which in turn may influence cell behaviour and consequently protein expression. The integrated study of the expression and activation of multiple proteins and signaling pathways provide powerful classifiers and predictors in breast cancer research. In this study, a proteomic screening of a significant number of breast cancer tissues, followed by a gene ontology study, has been performed, in order to identify novel proteomic clusters potentially involved in breast cancer progression. Our analyses highlighted three leading-clusters, (programmed cell death, glycolisis and cell motility), expressed in a high level in breast cancer tissues compared to normal adjacent tissues[3]. The patient screening revealed that proteins involved in programmed cell death and glycolysis pathways are expressed in the large majority of them, suggesting, respectively, the cell survival-dependence for primary tumor growth and the altered metabolism of tumoral cells. Conversely, the unevenly expression of proteins involved in cell motility among the studied cases, implies the existence of heterogeneous metastatic potentialities among patients, and the possible clinical application of the motility cluster as prognostic factors. Conclusively, functional proteomic, applied to a large number of cases, improves the knowledge of the main-traits of breast cancer and offers new possibility to sub-classify patients for prognostic purposes.

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6-MERS HYALURONAN OLIGOSACCHARIDES STIMULATE INFLAMMATORY RESPONSE IN NEURONAL-LIKE DIFFERENTIATED SH-SY5Y NEUROBLASTOMA CELLS

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Low molecular weight HA elicited pro-inflammatory responses by modulating both toll-like receptor 4 (TLR-4) and CD44. CD44 and TLR-4 stimulation activates different inflammatory pathways that culminate with the activation of the transcriptional nuclear factor kappaB (NF-kappaB), which in turn is responsible for the expression of inflammation mediators such as tumor necrosis factor alpha (TNF-alpha), interleukin-6 (IL-6) and interleukin-1 beta (IL-1beta)[1,2]. Although, the pro- inflammatory effects of HA small fragments have been reported in several cell lines such as fibroblasts, chondrocytes and synoviocytes[2,3], little is known about such effects in neuronal cells. The aim of this study was to investigate the influence of short HA oligosaccharides (HA 6-mers) in the inflammatory response in neuronal-like differentiated SH-SY5Y neuroblastoma cells. mRNA and related protein levels were measured for: TLR-2, TLR-4, CD44, TNF-alpha, iNOS, IL-1beta, IL-6, and the matrix metalloprotease-2 and 9 (MMP-2 and MMP-9) in cells with and without the addition of HA 6mer. NF-kB activation was also evaluated. 6-mer HA treatment produced a significant up-regulation of all parameters considered, which is in line with the well-known pro inflammatory effect of low molecular weight HA. Experiments using small interfering RNAs (siRNAs) are in progress, with the aim to block TLR-2, TLR-4 and CD44 expression. The modulatory effects of such experiments will be discussed.

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