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Guest Editor
Mario Raspanti

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European Journal of Histochemistry
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INVITED LECTURE

ELECTRON TOMOGRAPHY OF COLLAGEN-PROTEOGLYCAN INTERACTIONS IN THE CORNEA

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Heterotypic Type I/V collagen fibrils within the cornea are embedded in a proteoglycan matrix that regulates fibril diameter and spatial order, both pre-requisites for corneal transparency[1]. The mechanisms by which proteoglycans precisely maintain fibril architecture are not fully elucidated, however. Through three-dimensional electron microscopy techniques, this investigation examined the morphological characteristics and organisation of proteoglycans, in normal and genetically altered mouse corneas, to further our understanding of proteoglycan structure-function relationships.

Initially, wild-type mouse corneas were studied to obtain a clear understanding of murine collagen-proteoglycan interactions and to compound previous studies on how proteoglycans are organised three-dimensionally in the corneal stroma[2]. A single axis, -60° - +60° tilt series was captured using a JEOL 1010 transmission electron microscope and aligned using IMOD before segmentation with EM3D. A disordered arrangement of proteoglycans around collagen fibrils was evident in the mouse cornea, which suggested a dynamic, fluid corneal stroma capable of maintaining an ordered collagen array whilst allowing for passive movement of water and nutrients through the tissue[3]. Mice lacking the gene *Chst5*, normally responsible for encoding an enzyme (N-acteylglucosamine 6-O sulphotransferase) which transfers sulphate residues onto keratan sulphate glycosaminoglycans at biosynthesis, were also studied. The Cuprolinic Blue complexed proteoglycans appeared to aggregate beyond the normal size of those present in wild-type mouse cornea, whilst they also exhibit near-periodic, orthogonal off-shoots[4]. Furthermore, three-dimensional reconstruction of homozygous lumican-null mice corneas highlighted the importance of keratan sulphate proteoglycans in the maintenance of collagen fibril architecture in the cornea.

In summary, we found that proteoglycans are primarily responsible for the remarkable collagen organisation in the mouse cornea, which allows for corneal transparency. The self-association of proteoglycans into complexes is likely to supply a robust attachment of neighbouring fibrils, whilst sulphation patterns are seen to have a direct effect on the aggregation potential of proteoglycans. Removal of proteoglycans, particularly lumican, affects the regulation of both fibril size and spatial order, both required for corneal transparency.

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ABSTRACTS

INFLUENCE OF RUNNING ON EXTRACELLULAR MATRIX OF RAT ACHILLES TENDON

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Exercise and physical activity improve health and efficiency of cardio-vascular system but exercise-induced effects on skeletal system or soft connective tissues such as tendons and ligaments are not clear. Some studies revealed no biochemical or biomechanical changes in exercised tendons, whereas most of them reported an increased tendon stiffness, a tendon hypertrophy and a higher content of collagen type III, glycosaminoglycans and tenocytes proliferation[1-3]. With the aim of studying a cell-direct extracellular matrix remodeling after a period of running, we investigated the metabolism and expression of tenocytes explanted from tendon of eight running (RUN) and eight sedentary (SED) rats after a progressive program of treadmill running training. Morphometric analysis of crimping pattern as an expression of a tendon functional parameter in the proximal, central and distal portion of other six rats Achilles tendons was also performed at the polarized light microscope. Sprague Dawley rats run 30 min. a day for 12 weeks at a constant speed (16 m/min). Tendon cells were isolated and after 3 and 7 days of culture the cells proliferation and cells bioactivity were valued. No difference in tenocytes proliferation, collagen type II, elastin and fibronectin synthesis were observed between RUN and SED rats, both at 3 and 7 days. Also IL-1β, IL-6 and TIMP-1 didn't show differences between RUN and SED groups, thus indicating no inflammatory processes were present in all animals. TNF and MMP-13 (-37,84%) were even lower in RUN group vs. SED group. Differently TGF β 1 (+68,7 %), collagen type I (+14,54%), proteoglycans (+87,86%) and decorin (+19,29%) were higher in RUN vs, SED animals. Moreover all the analyzed parameters showed an obvious time-related decrease, from the 3 to the 7 days cultures, with the exception of cell proliferation, collagen type I synthesis, proteoglycans and decorin production. Morphometric analysis of crimping pattern showed that running training reduced the top angle width of crimp in the proximal and distal regions of Achilles tendon, whereas they were fewer, more flattened and with a larger base-length in the central portion of Achilles tendons of RUN animals. All changes in cells bioactivity and crimping pattern of RUN tendons appear as a tissue adaptation in response to running training.

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EXTRACELLULAR MATRIX NANO-MECHANICS DETERMINE MEGAKARYOCITE FUNCTION

A. Malara[1], C. Gruppi[1], I. Pallotta[1,4], E. Spedden[2], R. Tenni[1], M. Raspanti[3], D. Kaplan[4], M.E. Tira[1], C. Staii[2], A. Balduini[1,4]
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Cells feel the surrounding environment through integrin

receptors. Contact with extracellular matrix proteins (ECMs) leads to activation of specific biochemical signaling pathways and to cytoskeletal modifications that regulate processes such as cell differentiation, migration and apoptosis. Recent findings have demonstrated that mechanical properties of ECMs play an important role in determining cells behavior during these processes. While integrin ligation is important for signaling, mechanical tension has been demonstrated necessary to keep these intracellular signaling. In the bone marrow, ECMs concur to the generation of cues that are important for hemopoietic stem cells maturation and differentiation. Endosteal bone and vascular districts have been proposed as critical niches for stem cell differentiation into the megakaryocytic lineage. Differences in ECMs composition, physical state and stiffness may affect all the steps that lead HSCs to differentiate into Megakaryocytes (Mks).
In this work we have used a chemical modified collagen that completely override *in vitro* collagen ligand pathways in directing Mks response in term of cell spreading, migration, platelet release and fibronectin assembly. This different behavior seems to be related to the different nanomechanical properties of modified collagen with respect to native protein. N-acetylation of lysine side chains in collagen blocks the formation of banded fibrils and its self-aggregation leading to differences in the sopramolecular organization of this protein *in vitro*. AFM analysis of Mks interaction with this modified collagen clearly demonstrated that absence of fibrils, despite similar integrin engagement, and different mechanical properties in these proteins, regulate Mks behaviour and fate. In conclusion this study demonstrate that ECM structure and in particular collagen fibers are responsible for determining biological processes in human megakaryocytes, such as cell spreading, cell migration, platelet release and fibronectin assembly that are strictly dependent on cell contractility. New insights into signaling pathways and in mechano-sensing systems of cells need to be addressed but nanoscale mechanical properties of ECMs seem to have an important role in regulating megakaryocyte behavior *in vitro* and probably *in vivo*. Modification of ECM structure within bone marrow in diseased state such as myelofibrosis could represent an example of ECMs mechanics derived aberrant megakaryopoiesis.

BIOLOGICAL ROLES OF IDURONIC ACID-CONTAINING DOMAINS IN AORTIC SMOOTH MUSCLE CELLS

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Chondroitin sulfate (CS), dermatan sulfate (DS), and CS/DS copolymer chains are biologically active[1,2]. Hallmark of DS is the presence of L-iduronic acid in place of D-glucuronic acid, which dictates some of the structural and biological features of the chain. CS/DS exist at the cell surface and in the extracellular matrix in the form of proteoglycans. We have identified two enzymes, dermatan sulfate epimerase 1 and 2 (DS-epi1 and DS-epi2)[3,4] which are needed for dermatan sulfate formation, and generated a mouse lacking the DS-epi1[5] in order to investigate in the *in vivo* roles of iduronic acid containing structures. The depletion of DS-epi1 leads to 90% reduction of iduronic acid residues in dermatan sulfate, resulting in skin fragility and altered collagen fibrils[5]. To better elucidate potential role of such iduronic acid containing domains, aortic smooth muscle cells from wild-type (WT) and DS-epi1 knock-out (KO) mice were isolated and tested *in vitro*. DS-epi1 KO cells are more spread in culture compared to WT. Although no difference in proliferation and senescence was detected, the KO cells showed a slower migration rate when compared to WT in a scratch assay. Preliminary data highlighted that KO cells differ in leading edges and in cytoskeleton organization during migration. Moreover, DS-epi1 KO cells adhered less to different substrates in attachment assay. This may be related to a change in integrin expression, as shown by qPCR data. These findings suggest an influence of cell surface iduronic acid containing proteoglycan(s) on focal adhesion complex formation and remodeling in aortic smooth muscle cells.

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ANALYSIS OF GROWTH PLATE SULFATION AND CELL PROLIFERATION IN A MOUSE MODEL WITH PROTEOGLYCAN UNDERSULFATION

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The sulfate transporter DTDST, also known as SCL26A2, is an ubiquitous expressed sulfate chloride antiporter of the cell membrane, crucial for the uptake of inorganic sulfate, that is required for proteoglycan sulfation. Mutations in SCL26A2 gene cause cartilage proteoglycan undersulfation resulting in a family of chondrodysplasias with different clinical outcomes from mild to lethal[1]. We studied the role of proteoglycan sulfation on long bone growth, in the growth plate (GP) of the dtd mouse, a mouse model that reproduces the essential aspects of the DTD phenotype in humans[2]. HPLC analyses show significant undersulfation of whole dtd growth plates compared to wild-type animals. The morphology of dtd GP was altered especially in the proliferative and hypertrophic zones, compared to wild-types, as well as the chondrocyte proliferation rate, measured by bromodeoxyuridine labeling. Immunohistochemistry combined with expression data of the dtd growth plate demonstrated that the sulfation defect alters the distribution pattern, but not expression, of Indian hedgehog, a long range morphogen required for chondrocyte proliferation and differentiation[3]. In order to investigate more in detail the Ihh signaling pathway, real-time RT-PCR analysis of samples from microdissected GPs showed an increase > 2 fold of the expression of Gli1 and Gli2, the positive effectors that control the expression of cyclin D1 and Wnts genes. The analysis of the Wnt/β-catenin pathway, that again target cyclin D1 gene, showed several upregulated genes in the dtd mouse. Surprisingly, cyclin D1 was upregulated in dtd mouse, in spite of the reduced proliferation rate of their chondrocytes in the GP; indeed, upregulation of cyclin D1 and growth arrest has been reported by others in chondrodysplasias with FGFR3 defects[4,5]. Further analysis are in progress in order to investigate the role of Wnt family in the cell cycle and in dtd bone growth.
These data suggest that in dtd mice proteoglycan undersulfation causes reduced chondrocyte proliferation via the Indian hedgehog pathway, therefore contributing to reduced long bone growth.
This work was supported by grants from Fondazione Cariplo (2007) and PRIN (2009).

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EFFECT OF MECHANICAL VENTILATION AND FLUID LOAD ON THE EXTRACELLULAR MATRIX OF THE LUNG

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Although mechanical ventilation is an essential presidium in the operating room and in the intensive care unit, its effect of mechanical ventilation on pulmonary extracellular matrix has been poorly investigated[1].
Starting from the above considerations, to examine the behavior of lung matrix undergoing different ventilatory regimens and define the least invasive ventilatory strategy, we performed experiments using anaesthetized rats ventilated as following: a) spontaneous breathing for 4 hours; b) 4h mechanical ventilation at low or high tidal volume and zero alveolar pressure; c) 4h mechanical ventilation at low or high tidal volumes and 5 cmH_2O positive end-expiratory pressure. Other rats were ventilated as in points a) though c) and, received a 7 $ml \cdot kg^{-1} \cdot hr^{-1}$ intravenous saline infusion for 4h. At the end of experiments, gas analysis, respiratory mechanics and biochemical determination of glycosaminoglycans and cytokines in the lung tissue were performed. Additional rats were exposed to the same surgical preparation and ventilatory procedures described and processed for light microscopy analysis. Images were acquired and analyzed to measure for inter-alveolar septa thickness, alveolar radius and cross sectional area and the area of the peripheral tissue region at alveoli confluence.
Morphological analyses revealed that alveolar septa thickness and the area of tissue at alveolar confluence (alveolar corners) were not normally distributed and became normal after being transformed into their natural logarithms. While the ventilatory strategy *per se* did not affect septa thickness, saline infusion determined a significant ($p<0.05$) thickening of the interalveolar septa and, with the exception of group c, a significant enlargement of corner region.
This study demonstrates that the pulmonary tissue matrix can be damaged when exposed to excessive local tissue stress and/or strain. Fluid load and increased tissue hydration also induces matrix disorganization and mild edema, but also may trigger early matrix remodeling, thus revealing potentially protective towards lung injury.

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EFFECTS OF SINGLE POINT MUTATION IN TROPOCOLLAGEN DOMAINS FOR THE STUDY OF THE MOLECULAR BASIS OF OSTEOGENESIS IMPERFECTA

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Osteogenesis imperfecta (OI) is a genetic disease characterized by fragile bones, skeletal deformities and, in severe cases, prenatal death that affects more than 1 in 10,000 individuals[1]. We show by full atomistic simulation in explicit solvent that OI mutations have a significant influence on the mechanical properties of single tropocollagen molecules, and that the severity of different forms of OI is directly correlated with the reduction of the mechanical stiffness of individual tropocollagen molecules[2]. The reduction of molecular stiffness provides insight into the molecular-scale mechanisms of the disease. The analysis of the molecular mechanisms reveals that physical parameters of side-chain volume and hydropathy index of the mutated residue control the loss of mechanical stiffness of individual tropocollagen molecules[3]. We propose a model that enables us to predict the loss of stiffness based on these physical characteristics of mutations. This finding provides an atomistic-level mechanistic understanding of the role of OI mutations in defining the properties of the basic protein constituents, which could eventually lead to new strategies for diagnosis and treatment the disease. The focus on material properties and their role in genetic diseases is an important, yet so far only little explored, aspect in studying the mechanisms that lead to pathological conditions. The consideration of how material properties change in diseases could lead to a new paradigm that may expand beyond the focus on biochemical readings alone and include a characterization of material properties in diagnosis and treatment.

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AN IN-VITRO MODEL OF CALCIFICATION FOR THE STUDY OF THE OSTEOGENIC POTENTIAL OF ADULT HUMAN DERMAL FIBROBLASTS

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In order to investigate the calcification process in both physiological or pathological conditions, in vitro osteogenic assays are generally performed using bone-derived cells, bone-marrow-derived mesenchymal stromal cells or vascular smooth muscle cells[1]. In normal healthy individuals, mineral formation is limited to specialized tissues as skeletal bone and teeth, however, there are many disorders (*i.e.* diabetes, kidney diseases, atherosclerosis as well as genetic conditions) in which soft connective tissues undergo mineralization[2].
In the present study a calcification assay has been established by isolating dermal fibroblasts from adult individuals and by growing these cells in a calcifying medium in which DMEM has been supplemented with 10mM β-glycerophosphate, 50μg/ml ascorbic acid and 10 nM dexametasone. After different periods of culture, up to 40 days, fibroblast cell cultures were stained with the Von Kossa method and the activity of alkaline phosphatase (ALP) measured by a spectrophometric assay.
Results indicate that in-vitro human dermal fibroblasts, which are characterized by a limited life span in culture[3], are capable

to mineralize their secreted extracellular matrix, when grown in the presence of an osteogenic medium. Moreover, the process of mineralization appeared to progresses with time, since areas of calcifications become visible after two weeks of culture. Consistently with the activation of the osteogenic phenotype, fibroblasts exhibited also an upregulation of ALP activity. However, we have observed a remarkable heterogeneity among cells from different individuals, supporting the hypothesis that ALP is not a unique marker of calcification[4] and that the mineralization process is the result of a fine regulation of many inhibitors and stimulatory factors[5,6].
Work supported by grants from FCRMO(EctoCal).

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THE CORTICAL LAMELLAR BONE IN OSTEOGENESIS IMPERFECTA TYPE II STUDIED WITH LIGHT MICROSCOPY AND SCANNING ELECTRON MICROSCOPY

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The study was carried out on a population of 4 children affected by Osteogenesis Imperfecta (OI) type II aged from 5 to 15 years, who underwent osteotomy of femurs or tibias to correct severe bending of the long bones or for treatment of fresh fractures. The surgical procedure to correct the bending requires the resection of a segment of diaphysis, and these specimens were processed for polarized light microscopy and scanning electron microscopy. Equivalent specimens obtained by osteotomies carried out in 4 children age from 5 to 15 years affected by orthopaedic pathologies different from OI were used as controls. All specimens of cortical bone consisted in a complete transversal section of the diaphysis.
The controls presented with a regular circular or oval band of compact bone, while the O.I. were severely deformed or flattened with scattered masses of bone. The bone matrix was still lamellar but with a significantly lower number of secondary osteons. Other differences were observed in the size of the osteons and in the mean number of lamellae per osteon, while there were no differences between the mean lamellar thickness in the two populations (OI vs controls).
The systems of periosteal and endosteal lamellae were more evident in the OI cortical bone and this could be interpreted as an effect of the diaphyseal bending. Further research are needed to explore the relationships between the primary defect of the matrix and the load mechanism controlling the lamellar organization of the cortex in a severely deformed bone.

MORPHOLOGICAL ALTERATIONS OF DECIDUOUS TEETH WITH DENTINOGENESIS IMPERFECTA IN CHILDREN WITH OSTEOGENESIS IMPERFECTA: PRELIMINARY RESULTS

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Osteogenesis imperfecta (OI) comprises a heterogeneous group of hereditary disorders of connective tissue characterized by brittle bones with frequent fractures, osteopenia, ipostatural, progressive skeletal deformities, blue sclerae, dentinogenesis imperfecta, ligamentous laxity and hearing loss. The dentinogenesis imperfecta (DGI) is transmitted as a dominant hereditary abnormality, which may manifest alone or combined with OI and occurs in 5-15% of cases being more common in OI type III and rarer in OI type I. DGI plays a role of great interest for the complexity of clinical problems related to it.
Aim: The aim of this study was to assay the structural and histo-morphological changes in hard tissue compartments of deciduous teeth in patients with OI and DGI, by means of current, innovative and highly specific methods.
Materials and Methods: Four children (6-8 years average age) with diagnosis of OI –type III at the Pediatric Orthopedic Unit of the Civil Hospital of Brescia were examined for dental alterations referable to DI. Each patient underwent a clinical and radiographic examination of the oral cavity in order to assess the presence of alterations in the enamel-dentin complex related to DGI. The examination of the oral cavity revealed for all children clinical aspects related to DGI. From these patients 10 deciduous teeth, extracted or normally exfoliated, were subjected to histological analysis with hematoxylin-eosin staining and immunohistochemistry examination (optical microscopy, FEI-SEM/TEM analysis) to investigate alterations in the dentin structure.
Results: Histological examination of deciduous teeth showed severe pathological changes in dentin structured into four different layers. A collagen defect due to odontoblast dysfunction was theorized to be responsible for the histological changes.
The morpho-histological examination showed the following characteristics:
- enamel not impaired in the presence of a normal layer of mantle dentin
- hypocalcified dentin showing several interglobular abnormal
- circumpulpal dentin with altered structure and decreased number of tubules characterized by abnormal orientation
- reduced size of the pulp chamber and root canal in presence of calcifications
- presence in the dentinal tissue of high water content and low content of inorganic components.

Conclusions: The alterations of dental hard tissues highlighted in this study confirmed the correlation between OI and DGI. The extent of morphological knowledge in the field can provide useful information for diagnosis and treatment of teeth with DGI in patients with OI, adding new knowledge and perspectives on the treatment of dentinogenesis imperfecta and teeth affected by this disease.

ULTRASTRUCTURAL MORPHOLOGY OF OSTEOBLASTS. A SCANNING ELECTRON MICROSCOPY STUDY OF THE ENDOSTEAL SURFACE

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The endosteal bone lining cells are a population mainly formed by osteoblasts and a few clusters of osteoclasts. Apparently the osteoblasts are similar in dimension and shape but it is well know that they differ in their final destination and in the way of maturing in osteocytes, or carrying on the lamellar deposition.
In order to better investigate the possible differences in in external and intracytoplasmic morphology we applied the osmic maceration technique (Pazzaglia *et al.* 2010) to endosteal surface of rabbit long bones.
The femurs and tibias from 6 male New Zealand white rabbits (about 8 months of age) were obtained immediately after death and freed from the soft tissues. The diaphysis were separated from the extremities with an hand saw and the obtained cylinders were split longitudinally in two hemidiaphyses. The marrow was gently removed by irrigating with cacodilate buffer with a syringe. The specimens were fixed in buffered glutaraldehyde solution (2 %) for two minutes and postfixed in a solution of osmium tetroxide 1% and potassium ferrocyanide 1.25% for two hours. The specimens were then immersed in a solution of 0,1% osmium tetroxide in PBS for 48 hours at room temperature. They were dehydrated in ascending grades of ethanol, subjected to critical point drying in CO_2, coated with 10 nm of gold in a vacuum sputter and studied with a Philips XL30 SEM.
After a first observation of the cell population lining the endosteal surface the specimen was removed from the SEM and, under the control of a stereoscopic microscope, a strip of adhesive tape was gently pressed against the endosteal surface. The tape stripe was then removed and positioned with the free adhesive surface on another stub, gold coated and examined with the SEM.
With this approach it was possible to easily observe the marrow and bone surfaces of osteoblasts and to correlate them to the shape and the position of intracytoplasmic organelles

Pazzaglia U.E., Congiu T., Marchese M., Dell'Orbo C. The shape modulation of osteoblast-osteocyte transformation and its correlation with the fibrillar organization in secondary osteons: a SEM study employing the graded osmic maceration technique (2010) Cell Tissue Res., 340 (3): 533-540

PATTERNS AND LAMELLAR ORGANIZATION OF SECONDARY OSTEONS IN HUMAN BONE

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The lamellar architecture of secondary osteons has been studied with Scanning Electron Microscopy (SEM) in transverse sections of human cortical bone. Na_3PO_4 etching was used to highlight the interface between neighbouring lamellae and improve the precision of measurements. These technical improvements allowed a morphometric, quantitative test of earlier assumptions concerning the lamellar thickness and revealed the existence of different lamellar patterns.
The main lamellar thickness resulted higher and with a wider range of variation in respect to earlier determinations. The number of lamellae showed a direct correlation with the matrix osteonal area and their thickness had a random distribution for osteonal size classes. The circular, concentrical pattern was the more frequently observed, but also spiral and crescent moon-shaped lamellae were documented.
Selected osteons were examined either with SEM or with polarized microscopy comparing corresponding sectors of the osteon with the two microscopic techniques. The bright bands in polarized light corresponded to the grooves of SEM Na_3PO_4 etched sections and the dark bands to the lamellar surface with the fibrils approximately oriented along the central canal axis. However also lamellae with large and blurred bright bands could be observed which did not have a clear correlation in SEM. These findings are in contrast with the assumption that all the fibrils layers within a lamella are oriented along a constant and unchangeable angle. We explained the different lamellar patterns with the synchronous or staggered recruitment and activation of the pool of osteoblasts committed to osteon completion.

INSIGHT ON STRUCTURAL AND KINETIC PROPERTIES OF HUMAN PROLIDASE AND ITS PATHOLOGICAL VARIANTS

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Prolidase (E.C.3.4.13.9) is the the only Mn II dependent intracellular dipeptidase able to cleave the X-Pro, X-Hyp imidodipeptides, thus being very important in the final stages of protein catabolism and collagen turnover. Mutations in the prolidase gene cause prolidase deficiency, a rare autosomal recessive connective tissue disorder.
We generated WT and mutated recombinant human prolidase in *E.coli*. We selected three mutations previously characterized in our patients: 231delTyr, Glu412Lys, Gly448Arg and causing, in homozygous condition, the lost of enzymatic activity. Tyr 231 is located at the N-term end in an helix region, Glu412 is involved in the metal binding and Gly448 is very close to it. Mutated recombinant enzymes showed very low catalytic efficiency respect with WT. Glu412Lys showed a low affinity for the substrate and a reduced V_{max}; Gly448Arg a normal affinity but a very low V_{max}, whereas 231delTyr had a higher K_M associated with a mild reduction of the V_{max}. The affinity constant for the cofactor Mn II was similar to WT in Gly448Arg, reduced in 231delTyr, higher in Glu412Lys. ICP-MS analyses detected one Mn II ion in the WT active site following extensive dialysis[1]. Interestingly two Mn II ions were detected in the Glu412Lys, suggesting that the nitrogen ligand coordinates the metal more strongly causing a rigidity in the active site. CD analysis did not reveal any significant alterations in the secondary structure of the mutant proteins, but changes in the tertiary structure were demonstrated by limited proteolysis and by fluorescence spectroscopy. Interestingly western blotting experiments on patients fibroblasts lysates revealed a reduced prolidase amount that, associated with normal mRNA levels, supported the presence of a structural alteration for 231delTyr and Gly448Arg. A delay in the dimerization process was detected in all mutated proteins; in particular Gly448Arg stayed in solution as a monomer.
A mouse model for prolidase deficiency, carring 4bp deletion in ex 14 of the PEPD gene, is now available in our laboratory. The mice showed reduced prolidase activity, darkened coat color, small body size, dark stained urine and skeletal abnormalities. The model will be used to further characterize the role of the prolidase and proline metabolism in PD pathophysiology as

well as to test therapeutic approaches for the treatment of the disease.

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REARRANGEMENTS OF THE *ABCC6* GENE IN ITALIAN PATIENTS WITH PXE

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Pseudoxanthoma elasticum (PXE), characterized by calcification and fragmentation of elastic fibers, is an autosomal recessive disorder, which is due to mutations in the *ABCC6* gene (chromosome 16p13.1). It did not present any obvious correlation with the extracellular matrix[1,2,3]. This gene is characterized by many repetitive elements (Alu sequencecs) in all introns and in the flanking genomic sequence, which are thought to be the cause of major rearrangements of the gene as demonstrated by the deletion of exons 23_29 and deletion of exon 15[4].

The resequencing of *ABCC6* gene in 214 Italian PXE patients revealed that: 79% were carriers of two mutations (either in homozygosity of compound heterozigosity) (group A). Among the other patients 13,5% were found to carry a single mutation; 4% were apparently homozygous for a nucleotide substitution not confirmed by family segregation analysis; whereas 3,5% had not point mutation detected by sequencing (GroupB) .

The objective of this study was to search for the deletion of exon 24 and exons 24-27 that had been previously reported in Italian PXE patients. To detect these deletions we developed a long range PCR procedure using appropriately designed primers.

Two unrelated patients, heterozygous for missense mutations, were found be carriers of the exon 24 deletion; two unrelated patients, heterozygous for missense or nonsense mutations, were heterozygous for the exons 24_27 deletion. This observation suggests that these two deletions are frequent in the Italian population.

The present and previous findings indicate that in PXE Italian patients the ex 24 deletion was found in 1%, the deletion of exons 23_29 was found present in 10% of cases while the deletion of exons 24_27 in 2% of cases. In 1% of patients the deletion involved the whole *ABCC6* gene.

The search of major deletions is highly recommended in PXE patients in whom the sequencing of *ABCC6* gene had revealed a single point/minute mutation in heterozygosity or in apparent homozygosity.

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THE INHIBITION OF HYALURONAN DEGRADATION REDUCED PRO-INFLAMMATORY CYTOKINES IN MOUSE SYNOVIAL FIBROBLASTS SUBJECTED TO COLLAGEN-INDUCED ARTHRITIS

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Hyaluronan (HA) degradation produces small oligosaccharides that contribute to cartilage damage in arthritis. HA fragments increase pro-inflammatory cytokines level in rheumatoid arthritis synovial fibroblasts (RASF) by activating both CD44 and the toll-like receptor 4 (TLR-4)[1,2]. CD44 and TLR-4 stimulation in turn activate the NF-kB that induces the production of pro-inflammatory cytokines. Degradation of HA occurs via two mechanisms: a non enzymatic one exerted by reactive oxygen species (ROS) and an enzymatic one controlled by different enzymes in particular hyaluronidases (HYAL1, HYAL2, and HYAL3)[3].We aimed to investigate the effects of inhibiting HA degradation (which prevents the formation of small HA fragments) in synovial fibroblasts obtained from normal DBA/J1 mice (NSF) and from mice (RASF) subjected to collagen induced arthritis (CIA), both fibroblast types stimulated with tumor necrosis factor-alpha (TNF-alpha).

TNF-alpha stimulation produced high mRNA expression and the related protein production of CD44 and TLR-4 in both NSF and RASF, and activation of NF-kB found in all fibroblasts.

However, the over expression of the two receptors was higher in RAFS than in NSF.

A significant increase of inflammatory cytokines level, interleukin-1beta (IL-1beta) and interleukin-6 (IL-6), and other pro-inflammatory mediators, such as matrix metalloprotease-13 (MMP-13) and inducible nitric oxide synthase (iNOS) was consequently assayed. In particular, TNF-α treatment induced an inflammatory response more intensely in RASF than in NSF.

Treatment of NSF and RASF with antioxidants and specific HYAL1, HYAL2, and HYAL3 small interference RNA (siRNAs) significantly reduced TLR-4 and CD44 over-expression as well as the release of inflammatory mediators up-regulated by CIA.

These data mean that the treatment of NSF and RASF with TNF-α activated inflammation through a series of mechanisms, including HYAL and ROS stimulation that in turn degraded native high molecular weight HA. The small HA produced fragments interacted with TLR-4 and CD44 and were thereby able to increase TLR-4 and CD44 expression and protein synthesis in both NSF and RASF.

These results suggest that the inhibition of HA degradation during arthritis may contribute to reducing TLR-4 and CD44 activation and the inflammatory mediators response.

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THE MESODERMAL PROTEOGLYCAN VERSICAN GUIDES THE NEURAL CREST CELLS MIGRATION IN XENOPUS LAEVIS EMBRYOS

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Versican, a main proteoglycan (PG) of the early embryonic extracellular matrix, is involved in embryonal cellular processes and molecular interactions, such as heart morphogenesis and neural crest cells (NCC) migration. NCC are multipotent, migratory embryonic stam cells that give rise to a diverse cell lineage including melanocytes, smooth muscle cells, peripheral neurons and glia. Although several studies have been performed to define the role of Versican during the NCC migration, the real function of this PG in this important embryonic process is still largerly unclear. Previously, to better understand this role, we cloned Versican gene in Xenopus laevis embryo[1], defined its spatio-temporal distribution1 and performed loss of function experiments. We found that Xenopus Versican is duplicated in two genes generating 6 alternative splicing isoforms (4 in higher vertebrates): two full-length V0 forms (V0-1, V0-2), and six variants (three V1 , two V2 and one V3)[2]. Expression analysis during the embryonic development has revealed an early expression of the full-length V0-1 and V1 and V3 variants for both genetic and protein levels[2]; whereas, loss of function experiments confirmed that Versican plays a crucial role in the NCC migration. In particular, Versican exerts a promoting effect on NCC migration in contrast to an inhibitory effect of other molecules such as aggrecan[3]. Finally, we investigated the interactions between Versican and NCC. Thus, to test whether Versican is able to interact directly with NCC as a product of NCC itself or as an external component of the embryonic mesoderm, we carried out in vitro migration assays, analayzed by time-lapse microscopy, by using NCC and mesoderm explants of both Versican knock-down and wild type Xenopus embryos. When Versican knock-down NCC explants were coltured in close proximity to the wild type mesoderm explants, NCC were able to invade the mesoderm; when wild type NCC explants were coltured in close proximity to the Versican knock-down mesoderm explants, we observed that NCC were unable to invade the mesoderm. These results unequivocally showed that Versican expressed in the embryonic mesoderm guides the NCC along the right pathways during their migration.

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HEPARANASE REGULATES EPITHELIAL-MESENCHYMAL TRANSITION (EMT) INDUCED BY FGF-2

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Heparanase (HPSE) is an endoglycosidase that cleaves heparan sulphate (HS) chains. It takes part in extracellular-matrix remodeling[1] and regulate the bioavailability of HS-bound growth factors such as FGF-2. The FGF-2-receptors interaction is regulated by HS-proteoglycans like syndecan-1 (SDC1) which could be considered either an inhibitor of the FGF-2 signaling, when anchored on the plasma membrane, or a potent activator when shedded. Our recent study showed that heparanase has the ability to regulate the expression of SDC1 in proximal tubular epithelial cells (PTECs)[2].

Several chronic kidney diseases are sustained by tubular fibrosis and the epithelial-mesenchymal transition (EMT) of PTECs is considered one of the pathogenic mechanisms. Moreover it has been shown that FGF-2 triggers EMT in PTECs[3].

In this study we aimed to demonstrate that HPSE is involved in FGF-2-induced EMT in PTECs.

We confirmed that FGF-2 produces EMT in PTECs increasing the expression of mesenchymal markers SMA, vimentin and fibronectin. Moreover FGF2 increases MMP9 expression and activity, sustains cell motility and triggers morphological changes. Differently, these events do not occur in a PTECs cell line stably silenced for HPSE, showing that the lack of heparanase makes these cells resistant to EMT.

We showed that FGF-2 induce EMT through the PI3K/AKT pathway and that this signaling is sustained by the presence of HPSE. At the same time, FGF-2 impairs the expression of SDC1 and increases the release of MMP9 and HPSE in the extra-cellular environment.

Overall, our results show that: 1) a basal HPSE expression is necessary to support FGF-2-signaling and hence HPSE-silenced cells are resistant to EMT induced by FGF-2; 2) FGF-2 produces an autocrine loop to sustain its signaling.

These data suggest that high amount of HPSE at the tubular level could create a milieu that favors FGF-2-induced EMT and hence HPSE may represent an interesting pharmacological target for the treatment of chronic kidney diseases.

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BEHAVIOURAL CHANGES OF BREST CANCER CELLS IN VITRO

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We have previously reported on breast cancer the occurrence of extensive fragmentation of pre-existing collagen fibrils and new deposition of thinner fibrils formed mostly by $\alpha 1(I)_3$ homotrimer collagen of type I[1,2], which appears preferentially produced by cancer cells[3]. More recently this collagen has been discovered to be resistant to collagenases, due to less efficient unwinding of homotrimers by MMP-1[4]. When neoplastic cells are exposed to this collagen in an *in vitro* system, they respond by increasing proliferation and migration rates, and display a proteomic and genomic differential profiling versus the control cell cultures. Concurrently, we observed an increase of type V collagen, that when used as a substrate for cell culture, induced opposite effects to that exerted by the homotrimer collagen. A similar restraining effect was obtained when cells were induced

to express decorin, a small proteoglycan lysine-rich[5]. More recently we have extended the proteomic analyses to identify new protein clusters differentially expressed in response to different collagen substrates. Proteomic modulation regarded primarily proteins involved in the regulation of cell cycle and apoptosis, cytoskeleton proteins, metabolic enzymes and chaperonins. Cell morphology assays showed remarkable cell surface modifications with spikes formation and release of membrane vesicles, which are at present object of collaborative studies[6]. These membrane shuffling are directed by cytoskeleton rearrangement, which is also involved in complex responses induced by extracellular clues. In conclusion, the present information based on the vitro studies of the breast cancer systems, strongly suggest that the neoplastic cells are still able to respond to a number of extracellular signals emerging from the microenvironment, which could be in future used to improve the clinical approach to the patients.

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AUTOANTIBODIES IDENTIFICATION IN BREAST CANCER SERA BY PROTEOMIC APPROACH

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The recognition that human tumors stimulate the production of autoantibodies against autologous cellular proteins called tumor-associated antigens (TAAs) has opened the door to the possibility that circulating autoantibodies (AABs) could be exploited as serological tools for the early diagnosis and management of cancer. Cancer-associated autoantibodies are often driven by intracellular proteins that are mutated, modified, or aberrantly expressed in tumor cells. Moreover, the AAB have advantages over other serum proteins as potential cancer biomarkers as they are stable, highly specific, easily purified from serum, and are readily detected[1]. Emerging evidence suggests that autoantibody signatures in each type of cancer might explaining the role of important pathways in the development of disease. The advent of novel genomic, proteomic, and high throughput approaches has accelerated the interest for the serum autoantibody repertoire in human cancers to discover candidate TAAs. In the present study we aimed to use a proteomics-based approach as a mean of identifying antigens that elicit a humoral response in breast cancer patients[2]. To this purpouse we set-up a mass-spectrometry-based method that uses native tumor proteins immunoprecipitated by the IgG autoantibodies obtained from autologous breast cancer serum and from cancer-free controls sera. Moreover, serological approach (SERPA) was used as method to confirm the identification of the AABs. Interestingly, we identified a subset of 14 autoantigens and different isoforms that were highly representative of proteins with roles in key processes in carcinogenesis and metastasis, such as metabolic pathway, response to stress, cell cycle regulation and apoptosis, up-regulated in breast malignant tissues from all breast cancer patients tested[3]. Finally, the occurrence of immunoreactivity of serum samples against recombinant human α-enolase was investigated in a cohort of breast cancer patients and normal controls sera.

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ANTITHETICS OF GLYPICAN FUNCTION IN SOFT-TISSUE SARCOMA PROGRESSION

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Cell surface proteoglycans (PGs), including syndecans (SDCs) and glypicans (GPCs), are involved in the regulation of a number of cellular phenomena associated with the biology of cancer, but their precise function in the different facets of cancer progression remains to be fully unveiled. Experimental evidence during the years, and our more recent findings, suggest that different PGs may exhibit counteractive functions. While PGs such as SDC2, SDC4 and GPC1 may promote tumourigenesis, GPC3 and GPC5 seem to inhibit progression of discrete tumours. Using soft-tissue sarcoma as a tumour model we strive at understanding if different combinations of cell surface PGs act in a synergic manner to favour tumour cell growth and dissemination, or act in an antagonistic manner to impede tumour expansion and metastasis formation. To address this rather complex issue, we are pursuing a systematic in vivo-in vitro strategy entailing the use of model cell lines with different constitutive PG expression patterns that we can modify by RNAi and gene transduction; tumourigenesis assays in wild type, immunodeficient and transgenic mice; and post-genomic and proteomic screens. A first line of investigation has focused upon the putative onco-suppressing role of GPC4 and GPC5 (while GPC6 was used as reference GPC). Enhanced expression of GPC4 and GPC5 strongly reduces local tumour growth and lung nodule formation in a xenogenic setting featuring athymic mice, whereas overexpression of GPC6 has no effect. GPC5, but not GPC4 or GPC6, impede anchorage independent growth and modulate cell shape. Each of the GPCs dictates a defined adhesive and motile behaviour in response to isolated ECM components. Both GPC4 and GPC5 preferentially concentrate in focal adhesions and undergo internalization by clathrin-mediated endocytosis. A global antibody-based phosphor-proteomic analysis is currently been adopted along with RNAi-mediated knockdown of the GPCs in high expressing cells to confirm the onco-suppressing role of the PGs and dissect the signal transduction pathways that may be responsible for the control of cell behaviour. In parallel, we modulate the background constitutive expression of SDC1 and SDC4 by RNAi to establish whether these co-expressed PGs may influence GPCs activity. Our findings strongly correlate with recent observations on the tumour suppressing role of GPC3 and GPC5 in lung cancer patients and similar correlations are being searched in soft-tissue sarcoma patients.

PHENOTYPIC PROFILING OF THYROID CARCINOMA CELL LINES AND DIFFERENTIAL SECRETION OF MATRIX METALLOPROTEINASES

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Thyroid gland is composed of follicular structures consisting in prevalence of thyrocytes and few C cells. Each follicle is supported by the stroma containing interfollicular extracellular matrix (ECM), a capillary network and a few stromal cell types such as fibroblasts and inflammatory cells[1].

The glandular ECM plays a primary role in the maintenance of thyrocyte polarity and homeostasis. The interfollicular matrix, as it is reported in other contexts, represents a dynamic structure due to a regulated remodeling of its components. Major responsible of matrix degradation are the matrix metalloproteases (MMPs), which belong to a large family of zinc-dependent endopeptidases. At present 23 members of the MMPs have been described in humans and classified into subsets of enzymes, according to their molecular domains and preference for the substrates. 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.

Likely, a deregulated proteolysis of ECM molecules in thyroid tumors may cause the alteration of follicular structure and the loss of cell polarity. These events, in turn, may induce neoplastic cells to elude cell-cell and cell-ECM adhesions, promoting cancer progression[2]. In general, these alterations are correlated with poor prognosis in many tumor histotypes, where a positive relationship between the increase of MMPs and the malignancy grade has also been observed[3].

To contribute to the knowledge of thyrode tumorigenesis, we performed a differential analysis of two thyroid cancer cell lines, derived respectively from a papillary (B-CPAP) and an anaplastic (8505C) thyroid carcinomas. The first is a benign form of tumour, while the second is very aggressive. We performed comparative assays on the two cell lines, based on scanning electron microscopy, proteomic profiling, gelatinase activities and motility assay.

Our results showed that the 8505C cells display a very aggressive *in vitro* phenotype, with respect to B-CPAP, regarding increased cell motility and MMPs secretion, as well as the loss of cell polarity and acquisition of pleomorphic cell shape and differential proteomic expression.

We suggest that present data may be useful for increasing the knowledge on the typization of these most recurrent thyroid tumors.

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INVITED LECTURE

BIOMATERIALS: FROM ARTICULAR PROSTHESES IN ORTHOPAEDICS TO RESORBABLE SCAFFOLDS FOR TISSUE ENGINEERING APPLICATIONS

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In 2009 Professor David F. Williams, Editor-in-Chief of *Biomaterials*, wrote *the situations in which biomaterials are currently used are vastly different to those of just a decade ago*[1]. Actually, implantable materials, as those for the production of articular prostheses, are still of great importance and are presently used in a wide number of clinical applications; moreover, many new biomedical technologies are under development, which do not only include traditional materials (metals, ceramics and synthetic polymers) but also biopolymers, self assembled systems, nanoparticles, carbon nanotubes and quantum dots. As a consequence, the original concepts of biomaterials have to be modified.

After defining what biomaterials are, giving some example of their current applications, this paper focuses the exploitation of innovative materials for tissue engineering purposes. Interestingly, while definition of tissue engineering (*the creation* (or formation) *of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells through a systematic combination of molecular and mechanical signals*) does not explicitly mention any particular material, it is widely acknowledged that tissue engineering techniques necessitate non-conventional biomaterials[1]. For example, considerable improvements in creating engineered constructs can be achieved by the use of peptides as components of biomaterials[2]. Many attempts have been recently proposed to realize biomimetic materials, which are often indicated as *bio-inspired materials*[3]. A critical review of the state-of the-art in the field of biomaterials science and applications will be illustrated.

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ABSTRACTS

TISSUE ENGINEERING AND REGENERATIVE MEDICINE IN 2020: LOOKING AT THE PRESENT TO IMPROVE THE FUTURE

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In 2020 is estimated that Chinese and Indian economies will worthly account for almost 40% of the total value. Billion people will have soon access to new resources at their first and are going to change their life style. As a consequence food consumption will increase and shift in dietary patterns with considerably health issues. While consumption of animal fats and sugars is declining in the western world, it is going to be introduced, even if at low levels, in developing countries[1].

In 2020, it is forecasted that medicine will move mainly towards treatment of general disease and disorders and num-

ber of intervention will dramatically decrease mainly for the reduced number of re-operations. Primary and secondary prevention will represent the best strategy to reduce the risk of surgery both in men and women. These desirable occurrences would be possibly due to tissue Engineering and Regenerative Medicine strategies that to date are being pursued for the development of viable replacement organs such as blood vessels, heart valves, cartilage, bones, esophagus, liver, contractile myocardium, patch materials in general, etc... Research in these fields already obtained some encouraging results but the challenge is still open.
The development of small and large animal models contributed largely, so far, to explore new insights into the regeneration mechanism of *in vitro* fabricated organs and tissues but within 2020, a realistic perspective, is represented by the use on a large scale of phylogentical model closer to humans, like primates, or eventually as well as by the development of humanized animal like to α-Gal knocked-out pigs already experimentally tested for heart and kidney xenotransplantation.
As instance it has been recently verified that, purified progenitor cells from Rhesus monkey ES cells engrafted into non human primate hearts, in which they differentiated into cardiac cells without forming teratomas. These findings move the field another step closer to clinical use of ES or iPS cell-derived cardiovascular progenitors in cardiac repair[2]. However even if many data highlighted the perspective to use stem cells from different sources in cardio-vascular tissue engineering many contradiction must still be solved. It is feasible to hypothesize that in 2020 medicine will be at the beginning of use stem cell in clinical practice.
A step beyond conventional scaffold-based tissue engineering is cell-based direct biofabrication techniques. In industrial processes, various three-dimensional (3D) prototype models have been already produced using several different rapid prototyping methods, such as stereo-lithography, 3D printing and laser sintering[3].
Bioprinting of organs and tissues, by means of computer-aided transfer processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization, is an emerging field suggesting a strong growth potential aimed at producing bio-engineered structures serving in regenerative medicine and pharmacokinetic[4].

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PHYSICAL AND STRUCTURAL CHARACTERIZATION OF BOVINE AND PORCINE PERICARDIA BEFORE AND AFTER TRITDOC DECELLULARIZATION PROTOCOL: EVALUATION OF BIOMATERIALS FOR TISSUE ENGINEERING OF HEART VALVES

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Among cardiovascular disease, heart valve replacement with mechanical and biological prostheses is the most frequent procedure, unfortunately often leading to complications or reoperations. Tissue Engineering (TE) investigates the possibility to create viable valve substitutes able to withstand in vivo mechanical stress and undergo remodelling and growth after implantation[1]. This study is aimed at the characterisation of physical, structural and mechanical propertiesof bovine and porcine pericardium, before and after a detergent-based decellularisation treatment (TRITDOC). The resulting acellular scaffolds are considered as experimental starting materials for TE, specifically for TE heart valve production. Pericardia of two different mammalian species (bovine and porcine) were explanted immediately after animal slaughter. For each pericardium, 4 anatomical areas have been identified and in each area of untreated (NT) and TRITDOC treated bovine and porcine pericardia the average thickness, density and water content were measured. Histological, immunological and ultrastructural analyses were performed. The decellularised bovine pericardium (BP) displayed a significant decrease of water content and density increase ($p<0.05$) compared to the NT parent samples. Moreover, in TRITDOC treated porcine pericardium (PP) only two areas showed a significant decrease in water content ($p<0.05$), compared to parent NT samples. Structural analyses demonstrated complete decellularisation in both BP and PP with matrix architecture grossly maintained. Collagen fibres displayed a different birefringent color pattern when observed after TRITDOC treatment under polarized light compared to the NT samples. One pericardial area among the 4 considered resulted to be the most homogeneous regarding the physical features analysed. Thus, the mechanical features of this region were analysed by Bose ElectroForce System, considering the orientation of the sample and the effect of the decellularisation procedure.
TRITDOC treatment represents a promising and appealing method for the production of an acellular scaffold of both porcine and bovine pericardia derivation. This investigation allowed us to identify some pericardial areas of potential interest for TE. Further studies should be carried out, particularly to assess the mechanical performance of the decellularized scaffolds.

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QUANTITATIVE EVALUATION OF THE ALPHA-GAL XENOANTIGEN IN CURRENTLY HEART VALVE BIOPROSTHESES. COMPARISON WITH THE ALPHA-GAL AMOUNT IN THE ORIGINAL TISSUES UTILIZED FOR THEIR FABRICATION BEFORE AND AFTER DIFFERENT DECELLULARIZATION PROCEDURE

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The glutaraldehyde fixed bioprostheses employed in cardiac surgery, exhibit in the long term dystrophic occurrences, mainly related to cusps degeneration[1]. The tissue engineered constructs have not yet been shown to grant a complete immunogenic tolerance, with the onset of inflammation[2,3]. Despite the glutaraldehyde fixation or decellularization procedures, these degenerative-inflammatory processes seem to be triggered by the persistent presence of reactive xenogenic residuals; a primary role in inducing such reactions is represented by the alpha-Gal antigen[4,5].
The aim of the study is to quantify the presence of such epitope in glutaraldehyde fixed xenogenic bioprostheses through the optimization of an innovative ELISA immunoassay[6]. The

amount of epitope in different commercially valve substitutes was compared with that present in native original tissue, before and after different decellularization treatments.
Porcine aortic and pulmonary valves, porcine and bovine pericardium and 5 different models of bioprosthetic valve are analyzed. The porcine valves were subjected to the immunological test before (native) and after 3 different detergent-based decellularization treatments. Samples of α1,3GalT knockout pig pericardium were analyzed before and after glutaraldehyde fixation in order to validate the test for its use on commercial bioprostheses.
The ELISA immunoassay was shown to recognize and quantify the amount of alpha-Gal xenoantigen both on native/decellularized tissues and after glutaraldehyde fixation. The bovine pericardial bioprostheses showed an average persistence of 24% ± 2,3 of the original xenoantigens, that become 47,5% ± 3,6 for the porcine counter parts. The results obtained from the decellularized valves showed the complete removal of xenogenic antigens through the use of the TRICOL method, while the DOC and DOC-SDS methods showed a preservation of the 56,2% ± 6,5 of the initial epitopes.
For the first time it was possible to determinate and quantify the presence of the alpha-Gal xenoantigen on glutaraldehyde fixed tissues such as heart valve bioprostheses currently used in clinical settings. The study also provided evidence of the effectiveness of different decellularization treatments currently proposed and/or applied for the production of tissue engineered heart valves.

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