UNIVERSITÀ DEGLI STUDI DI PAVIA
DIPARTIMENTO DI BIOCHIMICA *"A. Castellani"*

XXVIII Meeting of the Italian Society for the Study of Connective Tissues (SISC)

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6-7 November 2008

Università degli Studi di Pavia

Collegio Cairoli, Piazza Collegio Cairoli 1- Pavia

Con il patrocino di:

Università degli Studi di Pavia

Facoltà di Medicina e Chirurgia

Facoltà di Scienze MM. FF. NN.

Facoltà di Farmacia

Si ringraziano per la collaborazione:

Collegio Universitario Fratelli Cairoli - Università di Pavia

Associazione ex Alunni Collegio Cairoli

NOVEMBER 6th, 2008

14.00 - 15.00 **Registration**

15.00 - 15.30 **Opening Ceremony in memory of prof. Alessandro A. Castellani (1929 – 1988)**
Prof. Angiolino Stella (Rector of the Univerity of Pavia)
Prof. Alberto Calligaro (Dean of the Faculty of Medicine)
Prof. Lucio Toma (Dean of the Faculty of Sciences)
Prof. Amedeo Marini (Dean of the Faculty of Pharmacy)

TISSUE MINERALIZATION
Chair: C. Balduini, A. Ruggeri

15.30 – 16.15 CASTELLANI LECTURE
Biological Calcifications and Acid Proteoglycans: a New Solution to an Old Problem.
E. Bonucci (Roma)

16.15 – 16.30
Life of the Cavecells. Sketches of the Hard Tissues Populations
U. Pazzaglia, C. Dell'Orbo, D. Quacci, T. Congiu (Brescia, Varese)

16.30 - 16.45
On the Pathogenesis of Elastic Fiber Calcification in Patients with Beta-Thalassemia and with PXE-like Clinical Manifestations.
G. Annovi, F. Boraldi, R. Tiozzo, D. Guerra, P. Cianciulli, F. Sorrentino, G.L. Forni, I. Pasquali-Ronchetti, D. Quaglino (Modena, Roma, Genova)

16.45 – 17.15 **Coffee Break**

GENE EXPRESSION AND PROTEIN INTERACTIONS IN THE EXTRACELLULAR MATRIX
Chair: G. Cetta, A. Passi

17.15 – 17.30
Molecular Geometry of Collagen Fibril Anchorages to Decoran, Biglycan and Fibromodulin in Extracellular Matrices (ECMs).
J.E. Scott, J. Bella, A. Eid, O. Antipova, J.P.R.O. Orgel (Manchester, Chicago)

17.30 – 17.45
Gene Expression of *Xenopus Laevis* Versican Isoforms During Embryonal Development
S. Campo, G. Nastasi, A. Avenoso, A. D'ascola, P. Traina, P. Casini, M. Ori, I. Nardi, R. Perris, V. Parisi, G.M. Campo, A. Calatroni (Messina, Pisa, Parma)

17.45 – 18.00
Proteomic Characterization of Cultured Aged Human Dermal Fibroblasts
F. Boraldi, G. Annovi, R. Tiozzo, D. Quaglino (Modena)

18.00 – 18.15
Fibroblasts Enhance Proliferation and Invasion of Breast Cancer Cells (8701-BC)
P.Cancemi, M.R. Marabeti, N.N. Albanese, .F. Costantini, G. Di Cara, I. Pucci –Minafra (Palermo)

18.15 – 18.30
Studies on the Post-Translational Modification of Hyaluronan Synthases (HAS)
E. Karousou, M. Kamiryo, C.H. Heldin, P. Heldin (Uppsala, Varese)

18.30 – 18.45
Human serum albumin sulphydryl groups oxidation in plasma and atherosclerotic plaque extracts
A.J. Lepedda, A. Zinellu, A. Cigliano, E. Zinellu, G. Marongiu, F. Piredda, M.A. Casu, P.P. Bacciu, C. Carru, M. Formato (Sassari)

18.45 – 19.00
Direct GAG-Collagen Interaction: More Questions than Answers
M. Raspanti, C. Dell'Orbo, M. Viola, R. Tenni, M.E. Tira (Varese, Pavia)

19.00 – 20.00 SISC General Assembly

20.20 Social Dinner at Collegio Cairoli

NOVEMBER 7th, 2008

CONNECTIVE TISSUE DISEASES
Chair: A. Calatroni, I. Pasquali-Ronchetti

9.00 – 9.45 GOTTE LECTURE
Osteogenesis Imperfecta: Use of the BrtlIV Murine Model to Better Understand the Molecular Basis of OI and to Develop New Therapeutic Approaches
A. Forlino (Pavia)

9.45 – 10.00
Inflammation and Resolution in Cartilage: Involvement of Cox-2
V. Ulivi, R. Cancedda, F.Descalzi Cancedda (Genova)

10.00 – 10.15
Sulfation and Growth Parameters in the Epiphyseal Cartilage of a Murine Model of Diastrophic Dysplasia
M. Facchini, B. Gualeni, A. Forlino, F. Pecora, A. Icaro-Comaglia, F. Riva, M. Casasco, A. Calligaro, M. Viola, A. Passi, A. Lupi, R. Gioia, G. Cetta, R. Tenni, A. Rossi (Pavia, Varese)

10.15 – 10.30
ABCC6 Mutations in Italian PXE Patients: an Update Describing Twenty-Two Novel Mutations
D. Guerra, J. Roggiani, F. Panico, G. De Santis, D. Gheduzzi, D. Quaglino, I. Pasquali-Ronchetti (Modena)

10.30 – 10.45
Correlation between ApoE Polymorphism and Severity of Cardiovascular Manifestations in Pseudoxanthoma Elasticum (PXE)
I. Ronchetti, D. Gheduzzii, P. Tarugi, D. Guerra, J. Reggiani, F. Boraldi, G. Annovi, O. Vanakker, P. Couke, A. De Paepe, D. Quaglino (Modena, Gent)

10.45 – 11.15 Coffee break

NEW INSIGHT AND PERSPECTIVE IN BIOMATERIALS
Chair: V. Ottani, D. Quacci

11.15 - 11.30
New Insights into the Implant Osteointegration Techniques
A. Trirè, D. Martini, E. Orsini, V. De Pasquale, G. Giuliani Piccari, A.Ruggeri (Bologna)

11.30 – 11.45
Strategies to Modified the Material Surface with Calcified Matrix
E. Saino, V. Maliardi, L. Fassina, M. S. Sbarra, M. G. Cusella De Angelis, G. Magenes, F. Benazzo, L. Visai (Pavia)

11.45 – 12.00
Histomorphometric Evaluation of Implant Design as a Factor Positively Influencing Peri-Implant Bone Response. A Pilot Study in Dog Lower Jaw
E. Orsini, B. Bacchelli, A. Trirè, M. Macciocca, M. Quaranta, V. Ottani (Bologna)

12.00 – 12.15
Improved Cell Growth by Bio-Oss/Pla Scaffolds as a Bone Substitute
A. Asti, L. Visai, R.Dorati, B. Conti, E. Saino, M.S. Sbarra, G. Gastaldi, F. Benazzo (Pavia)

12.15 – 12.30
Physically Augmented Coating of Macro-Rough Titanium Surface with Human Saos-2 Osteoblasts and Bone Matrix
L. Fassina, E. Saino, L. Visai, G. Magenes (Pavia)

12.30 – 12.45
Differentiation of Adipose Tissue-Derived Stem Cells Cultured on Trabecular Titanium Scaffolds
A. Asti, G. Gastaldi, M.F. Scaffino, F. Benazzo (Pavia)

13.00 – 14.30 Lunch

STROMA AND CANCER
Chair: I. Pucci-Minafra, A. Ferlazzo

14.30 – 14.45
Tumor Microenvironment as Target in Cancer Prevention; Matrix Metallo-Proteases and Beyond
F. Fasano, I. Sogno, A.R. Cantelmo, R. Cammarota, D.M. Noonan, A. Albini (Milano, Varese)

14.45 – 15.00
Contribution of Connective and Epithelial Tissue Components to the Morphological Organization of Canine Trichoblastomas
G.A. Losa, G. De Vico, M. Cataldi, F. Carella, S. Beltraminelli (Locarno, Napoli, Messina)

15.00 – 15.15
S-100 Calcium Binding Proteins as Potential Markers for Breast Cancer Metastasis
I. Pucci-Minafra, P. Cancemi, G. Di Cara, N.N. Albanese, M.R Marabeti, F. Costantini, R. Musso, P. Taormina, A Marrazzo, S. Minafra (Palermo)

15.15 – 15.30
Anti-Oncogenic Role of Decorin: Identification of New Markers.
G. Di Cara, L. Minafra, P. Cancemi, N.N. Albanese, M.R. Marabeti, S. Feo, I. Pucci-Minafra (Palermo)

15.30 – 15.45
Mitochondriotropic Resveratrol Derivatives Cytotoxic to Fast-Growing Cells
L. Biasutto, A. Mattarei, A. Bradaschia, E. Marotta, M. Zoratti, C. Paradisi, S. Garbisa (Padova)

15.45 – 16.00
Multiple Myeloma Cell Aggressiveness Is Restrained by Hyperforin
V. Olivieri, S. Carraro, A. Bradaschia, C. Gattazzo, L. Trentin, A. Cabrelle, S. Garbisa (Padova)

16.00 – 16.30 Coffee break

ENDOGENOUS AND EXOGENOUS MODULATORS OF CONNECTIVE TISSUES
Chair: A. Rossi, D. Quaglino

16.30 – 16.45
Structure of Knee Medial Collateral and Patellar Ligament in Rat
M. Macciocca, M. Quaranta, A. Dionisi, L. Leonardi, M. Franchi (Bologna)

16.45 – 17.00
Chondroitin Sulphate, Heparan Sulphate And Hyaluronan Reduce Cytokine Production by Toll-Like Receptor-4 Modulation in Mouse Chondrocytes Stimulated with LPS
A. D'Ascola, A. Avenoso, S. Campo, P. Traina, A. Calatroni, G.M. Campo (Messina)

17.00 – 17.15
Endogenous Fibronectin Modulates Collagen-dependent Spreading and Proplatelet Formation by Human Megakaryocytes
A. Malara, S. Badalucco, L. Visai, C. Gruppi, R. Tenni, M.E. Tira, C. Balduini, A. Balduini, A. Pecci, M. Raspanti (Pavia, Varese)

17.15 – 17.30
Evaluation of Cellular Response of Breast Cancer Cells Grown on Distinctive Collagen Substrates.
N.N Albanese, P. Cancemi, G. Di Cara, M.R. Marabeti, F. Costantini, I. Pucci –Minafra (Palermo)

17.30 – 17.45
LDL Modulation of Hyaluronic Acid Secretion by Aortic Smooth Muscle Cells
B. Bartolini, M. Viola, D. Vigetti, E. Karousou, P. Moretto, M. Clerici, A.Genasetti, M. Rizzi, G. De Luca, A. Passi (Varese)

17.45 – 18.00
Energy Status of the Cell Regulates Hyaluronan Synthesis
D. Vigetti, M. Clerici, E. Karousou, M. Viola, M. Rizzi, B. Bartolini, A. Genasetti, P. Moretto, G. De Luca, A. Passi (Varese)

18.00 – 18.15
SPARC/BM-40/Osteonectin-Binding Sites on Fibrillar Collagens
C. Giudici, N. Raynal, H. Wiedemann, R. Timpl, H.P. Bächinger, R.W. Farndale, T. Sasaki, R.Tenni (Martinsried, Pavia, Cambridge, Portland)

18.15 End of the Meeting

BIOLOGICAL CALCIFICATIONS AND ACID PROTEOGLYCANS: A NEW SOLUTION TO AN OLD PROBLEM.

ERMANNO BONUCCI
Department of Experimental Medicine, La Sapienza University – Rome

The mechanism of biological calcifications is one of the most elusive problems in biology. In spite of a large number of investigations on the calcification of vertebrate and invertebrate mineralised tissues (for a review see Bonucci[1]), the established facts are very few. Among them, it has been definitively proved that biological calcifications do not take place through a precipitation mechanism, as it could occur in test tubes containing adequate amounts of calcium and phosphate or carbonate ions. The concentrations of these molecules in calcifying organic matrices are not, in fact, high enough to induce their spontaneous precipitation. Phosphate ions could be increased through the hydrolysis of phosphate esters catalysed by alkaline phosphatase, or indirectly through the production of ATP by glycolysis, but it has been shown that the inhibition of both these processes does not inhibit *in vitro* calcification. Actually, the biological calcifications are very complex processes that are regulated on one side by cells, which are indispensable for the exchange of electrolytes and the synthesis of the organic matrix, on the other by components of the organic matrix itself, one or several of which may have catalytic activity.

On the basis of these concepts it has been suggested that the collagen fibrils, which are the most abundant components of the bone matrix, may be the structures that catalyse the calcification process in bone[2]. As a matter of fact, transmission electron microscope (TEM) studies on this tissue show a very close relationship between the early inorganic crystals and the period of the collagen fibrils, to such a point that the latter seem to be negatively stained by the former. The inorganic substance, which is visible under the TEM because of its intrinsic electron density, behaves, in fact, as a negative dye: in the areas of early calcification, it localizes according to the periodic banding of the collagen fibrils and, more precisely, accumulates in their 'hole' zones. This observation has led to assume that atomic groups, located just in these zones, have the same spatial arrangement as that of the reticulum of the apatite crystals. This matching of lattice dimensions, which does not need to be necessarily perfect, induces the formation of early apatite inorganic nanoparticles – or nuclei – by epitaxy or by heterogeneous nucleation. The addition of further calcium and phosphate ions to the nuclei leads them to grow in size and to acquire the typical rod- or filament-like shape that characterizes the hydroxyapatite crystals in the fully calcified bone matrix[2,3].

These concepts were so convincing that they were accepted and taken for long as demonstrated. A series of incongruities, however, gradually developed and led to partly challenge the theory. Above all, it was contended that, if the rod- or filament-like crystals grow from inorganic nuclei within the fibrils, they need space to attain their final size and consequently they must open the cross-links between adjacent collagen molecules; the length of the definitive crystals is, in fact, greater than that of a collagen period and, consequently, of a hole zone. The formation of intrafibrillar crystals, therefore, should disrupt the fibril architecture and should increase the bone matrix solubility that, on the contrary, decreases during calcification. Moreover, it is necessary to consider that the collagen fibrils are components of few calcified tissues and that, of course, they cannot be directly involved in the calcification of non-collagenous tissues. It is unlikely, however, that different calcification mechanisms have developed during the biological evolution in different calcifying tissues (one tissue, one mechanism); on the contrary, it is plausible that the basic mechanism of the calcification process is always the same in all tissues. If this is true, collagen fibrils cannot be the calcification promoters common to all of them, because plenty of collagen fibrils are found in bone, dentin, cementum and calcifying tendons, but no significant numbers of collagen fibrils are recognizable in other hard tissues such as enamel, mollusc shells, crustacean integuments, or corals. For these reasons, other organic, non-collagenous components have been sought as possible mediators of inorganic crystal formation. They, in any case, might be constituents of, or might be associated with, the collagen fibrils in bone and other calcified collagenous tissues.

Most of the investigations aimed at finding the non-collagenous mediator(s) of the calcification process have been carried out on epiphyseal cartilage, a tissue less hard and easier to examine than bone. Among these researches, those carried out with the TEM have produced two main results[4]: first, the early crystals in the calcifying cartilage are located within structures of cellular origin called 'matrix vesicles' (reviewed by Sela et al.[5]) and are not in relationship with the collagen fibrils – which, by the way, are of type 2 while those of bone are of type 1; second, the early crystals themselves are not in relationship with collagen molecules, but with organic structures which have the same rod- or filament-like shape as that of the untreated crystals. Because these structures are only recognizable after the crystals have been removed by decalcification, they have been named "crystal ghosts"[4] (Fig. 1). The same structures have been found at the calcification front in bone, especially in woven bone, in which variable and sometimes prevalent amounts of crystals are contained in the interfibrillar spaces[6].

Actually, as shown in Fig. 1, the crystal ghosts are only recognizable in tissue specimens treated with the PEDS method (Post Embedding Decalcification and Staining)[4,7]. According to this method, the calcified specimens are firstly embedded in suitable resins

(Araldite, Epon, glycolmetachrylate) and are subsequently decalcified by flotation of the ultrathin sections on, or by immersion of the whole embedded specimens in, decalcifying solutions (usually EDTA or 1% formic acid). The decalcified sections are then stained with an electron-dense 'dye'. The rationale of this procedure is that all organic materials are immobilized by the resin and are consequently 'protected' from extraction during decalcification. It is known that, on the contrary, the organic components of the matrix, and specifically those of the calcified areas, are more or less completely extracted when decalcification is carried out before embedding. Also in this case, however, the not-embedded organic components can be more or less completely preserved by stabilizing anionic components with cationic dyes during fixation[8].

The structures called crystal ghosts have been found in all calcified tissues studied so far (reviewed by Bonucci[1,9]). They are easily recognizable in all calcifying areas in all hard tissues, whereas their evidence under the TEM decreases with increasing degree of calcification, to disappear in fully calcified areas. They have a so close resemblance to untreated crystals that the decalcified-stained areas may seem to be not decalcified (Fig. 1C). Actually, after the PEDS method, the calcified areas are completely decalcified as shown not only by their empty appearance (Fig. 1B), but above all by their von Kossa negativity and by their electron diffractograms of amorphous type. Even in tissues in which the calcified structures are not crystalline – like, for instance, the rosette-like inorganic
inclusions in mitochondria – the PEDS method shows rosette-like organic structures similar to the inorganic ones[10].

Because the crystal ghosts and the inorganic crystals have very similar ultrastructural shape, and because the crystal ghosts are found in all calcified tissues, it has been suggested that they could be the organic structures which nucleate the crystals in all tissues and that could represent a common calcification factor in all of them (reviewed by Bonucci[1,9,11]). Before considering this suggestion, examining thoroughly the role of crystal ghosts in calcification, and exploring their nature, it is necessary to refuse the idea that they might be due to technical artefacts[12,13].

First, the crystal ghosts are not, as hypothesized, crystals left undecalcified in the sections, because, as already discussed, they are only recognizable after staining and do not give electron diffractograms of crystal type; moreover, the decalcified areas are von Kossa negative. On the other hand, it is well know that even a few minute floating on distilled water induces complete decalcification of the ultrathin sections. In addition, their histochemical properties (see below) exclude that they are inorganic structures. Second, they are not, as suspected, clefts left in the resin by the removal of the crystals and filled by the staining solution, because they are visible in sections that have been re-embedded after decalcification. Moreover, structures similar to crystal ghosts are recognizable in sections from specimens that have been fixed in, and stabilized by, a cation solution (acridine orange) and have been decalcified before embedding[8]. Third, the crystal ghosts are not due to adsorption of organic material on the crystal surface, as supposed, both because in this case they should appear as allow cylinders, and as small rings in cross section, and because the method of preparation (fixation in phosphate buffered solution) would de-adsorb organic material from hydroxyapatite. On the other hand, biochemical analyses have shown that organic molecules are so-strongly linked to apatite crystals (so-called 'crystal bound proteins') that they can only be extracted after decalcification (see Bonucci[1,9] for further discussion). This introduces a fourth objection, connected with the spatial relationship between the organic and inorganic components: it has been objected, in fact, that, for crystallographic reasons, the crystal ghosts could not be contained 'inside' the crystals[14.] This objection appears a non-sense, because there is not an inside and an outside in nanoparticles that measure a few nanometers in thickness and that, at the early stage of calcification, are not yet true crystals. The structures that are found at the initial stage of calcification, and are conventionally but erroneously called crystals, as a matter of fact are organic-inorganic structures which give amorphous or very poorly crystalline electron diffractograms and are comparable to crystals only because of their rod- or filament-like shape. Their organic and inorganic components are intrinsically coupled, with formation of organic-inorganic hybrids. On the other hand, it has been repeatedly shown not only that organic material can be contained in biological crystals without disrupting their crystal lattice (reviewed by Bonucci[1,9]), but also that, in the case of the big calcite spicules of the sponges, the internalization of specialized proteic material allows organisms to control the growth of the crystals[15] and to increase their mechanical strength[16].

Established that the crystal ghosts are true structures and not technical artifacts, it is mandatory to look into their nature. To this aim, TEM studies have been carried out on the basis of previous findings showing that the calcified areas of the cartilage contain acid proteoglycans, as documented by their positive staining by Alcian blue and colloidal iron at low pH and by other histochemical methods[17.] A number of electron microscope histochemical investigations have been, therefore, carried out on the calcification front of epiphyseal cartilage and on the so-called calcification nodules, that is, the first areas of calcification. It has been shown that the crystal ghosts react in these areas with colloidal iron (at pH below 3), acidic phosphotungstic acid, ruthenium red and a number of cations (calcium, barium, magnesium, lanthanum, strontium, terbium chloride). Moreover, their reactivity is inhibited by methylation and incompletely and irregularly restored by saponification[18]. These and other histochemical findings (reviewed by Bonucci[9]) show that, at least in epiphyseal cartilage, the crystal ghosts pertain to acid proteoglycans (aggrecans) and, specifically, correspond to chondroitin sulfate[19]. These

conclusions are in full agreement with the old, but still actual, concept, reviewed several times[8,20,21], that, at least in cartilage, the acid proteoglycans, chiefly the chondroitin sulfate, have a principal role in the early stages of the calcification process. The conclusions on the nature of the crystal ghosts in cartilage are in full agreement with those of Sobel[22], inferred from results of light microscope histochemistry, that chondroitin sulfate, complexed with collagen in a critical configuration, is the 'local factor' that initiates the calcification process in cartilage. Actually, glycosaminoglycans can show a specific periodic interaction with collagen fibrils[23].

According to Sobel and Burger[24], sulfated proteoglycans are components not only of cartilage matrix, but also of that of bone, dentin and enamel, and can be found in areas of arterial ectopic calcification. The nature of crystal ghosts in calcifying, non-cartilaginous tissues remains, however, to be established. Several findings points to the conclusion that in all cases, as in the epiphyseal cartilage, the crystal ghosts are acidic polymeric molecules, the acidic groups ranging from sulfate groups of glycosaminoglycans and proteoglycans, to phosphate groups of phosphoproteins, to carboxylic groups of amino acids (reviewed by Bonucci,[1]). In this connection, aspartic acid is of particular interest. It is the most representative amino acid of the mollusc shell matrix proteins (30 mol % in the aragonite layer and more than 50 mol % in the calcite layer[25]); it has specific sequences that can induce the formation of early inorganic aggregates[26,27]; it can give rise to polyaspartate sequences in a β-sheet conformation, a pattern favourable to calcification because the spacing between aspartic acid residues comes to resemble the Ca-Ca distance in the 001 face of aragonite crystals[28].

In conclusion, the old idea that acid proteoglycans are the local factors that initiate calcification has found confirmation from TEM studies and ultrastructural histochemistry. Although the function of chondroitin sulfate might be confined to the epiphyseal cartilage, where its concentration is conspicuous, other acidic polymeric molecules might be responsible for the calcification process in other tissues. On the basis of the available results the following hypothesis can be put forward[1]: 1) acidic polymeric molecules of the organic matrix, whose structure and composition can change in different calcified tissues – and that in bone and other collagenous tissues might be associated with collagen fibrils – are unmasked and activated; 2) atomic groups of these molecules have the same spatial arrangement as those of the phospho- or carbonate-apatite; 2) this type of organic lattice can, therefore, link up and stabilize amorphous calcium phosphate or carbonate, so inducing the epitaxial formation of early apatite nuclei; 3) the nuclei grow by addition of other mineral ions and form chains of inorganic aggregates connected to the activated molecules; 4) the aggregates coalesce and originate solid, organic-inorganic hybrids that, as a whole, necessarily repeat the shape of the activated organic molecules (which consequently behave as templates); 4) the organic templates are gradually removed and the organic-inorganic hybrids "mature" into definitive crystals.

According to this hypothesis, the biological calcification would occur in all calcifying tissues through the same basic mechanism. This could be induced by activated atomic groups of organic molecules that, although different in different tissues, share the same general characteristics. They, in fact, in agreement with the supposed role of acid proteoglycans in epiphyseal cartilage, could be acidic, polymeric molecules in all cases. Because these molecules are genetically determined, the hypothesized process would also explain the formation and the maintenance of very complex architectural forms, which recur unchanged during the centuries, such as those of molluscs shells, echinoderm skeleton, crustacean cuticle, and many other multi- or unicellular organisms.

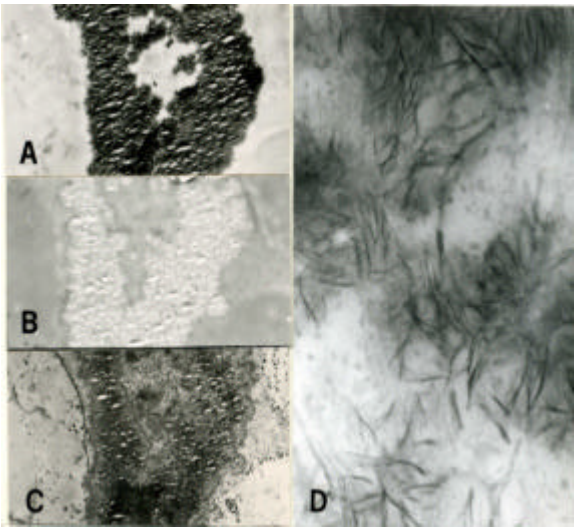

Fig. 1- A-C: three consecutive sections of the same calcifying area of epiphyseal cartilage, showing different stages of the PEDS method. x 7000. A) untreated section: the inorganic substance is strongly electron-dense (black), the surrounding uncalcified matrix shows low electron density. B) decalcified section: the inorganic substance has been removed and the previously electron-dense matrix now shows low electron density, lower than that of the surrounding uncalcified matrix. C) decalcified and U-Pb stained section: the decalcified area is now electron-dense (less than in A, but more than in B) and seems undecalcified. D) Detail of C, showing aggregates of crystal ghosts. x 60,000.

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LIFE OF THE CAVECELLS. SKETCHES OF THE HARD TISSUES POPULATIONS.

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Bone and cartilage are not static tissues. They undergo a significant turnover of a "solid" extracellular matrix in which cell function and mechanical properties are closely related to morphology. But, because of the matrix hardness, bone and cartilage are difficult tissues for microscopical studies, and the ultrastructural 3D morphology knowledge of their cells is related to application of few specific techniques. Often it is necessary, to obtain a good morphological result, to choose between hard and soft parts. We propose the use of the osmic maceration method coupled to Scanning Electron Microscopy to contribute to the 3D morphology of the bone and cartilage tissues. This approach allowed to represent entirely the soft tissues and cell population in bone and cartilage.

The bone specimen were split longitudinally with pliers in order to follow the natural fracture planes (see figure), determined by the lines of lower resistance in the mineralized matrix represented by vascular canals and osteocytic lacunae and canalicula. The fracture surfaces were treated in osmium ferrocyanide following a protocol in order to remove the solubile proteins of the matrix (Pazzaglia et al, in press).

Our technique showed an excellent visualization of the specimens; the internal surface of Havers' canals was lined by a texture of bundles of densely packed collagen fibers. The bundles were intertwined along the longitudinal axis of the canals and holes were regularly scattered between the bundles.

The differences between unmineralized and mineralized matrix were easy distinguishable in correspondence of vascular canal surface.

Capillaries wall, unmielinated nerves and pericytes were well exposed.

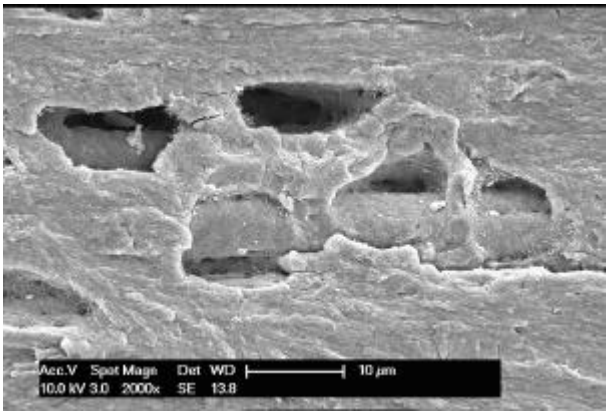
Osteoblasts showed processes extending to the unmineralized matrix and It was possible to follow the progressive entrapping in osteoid and becoming osteocytes.

The osteoclasts, easy identified by their ruffled borders, were visible in the cutting cones.

The osteocytes showed their long processes elongating inside the canalicula of the bone matrix. Osteocytic lacunae were lined by a texture of collagen fibers less densely packed than those forming the bundles inside canals and without any definite orientation.

The cartilage specimens were easy spliced with a razor blade. The texture of collagen bundles in the chondrocytic lacunae was well exposed revealing the pits corresponding to the cell prolongations, very similar to those of the ostecytes lacunae.

Further, the chondrocytes showed a good image of inner organuli architecture.



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ON THE PATHOGENESIS OF ELASTIC FIBER CALCIFICATION IN PATIENTS WITH BETA-THALASSEMIA AND WITH PXE-LIKE CLINICAL MANIFESTATIONS.

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It has been repeatedly reported that a number of β-thalassemia (beta-thal) patients manifest skin, eye and vessel clinical alterations almost identical to those typical of inherited Pseudoxanthoma elasticum (PXE) [1] Moreover, ultrastructural studies have shown that in both disorders the dermis exhibits identical alterations, such as calcification of elastic fibers, deposition of abnormal matrix constituents in the extracellular space and abnormal collagen fibrillogenesis [2]. Whereas inherited PXE is due to mutations in the ABCC6 gene encoding for the membrane transporter MRP6, on the contrary, analysis of the ABCC6 gene in a number of beta-thal patients, either with (PXE+) or without (PXE-) PXE-like manifestations, did not reveal any mutation [3]. Therefore, it would seem that PXE-like clinical manifestations in some beta-thal patients might be the result of abnormalities affecting the same metabolic pathways as in inherited PXE leading to similar extracellular matrix alterations.Recent studies have shown that fibroblasts from PXE patients suffer from a mild chronic oxidative stress [4] similarly to what has been already demonstrated for β-thal patients [5]. Moreover, we have recently shown by 2D gel electrophoresis and Western blot on whole cellular protein extracts that dermal fibroblasts from PXE patients exhibit reduced expression of carboxylated Matrix Gla Protein (Gla-MGP) [6]. Interestingly, MGP has an inhibitory role on the mineralization process of soft connective tissues, only if adequately carboxylated on its glutamic residues by a vitamin k-dependent system. Aim of the present investigation was to perform a 2DGE analysis of the protein profile of fibroblasts from beta-thal- PXE+ and beta-thal-PXE- patients and to compare data with those of fibroblasts from genetic PXE and healthy controls. We presented data demonstrating that in beta-thal-PXE+ and in PXE patients, but not in control and beta-thal-PXE- patients, there is a significant up-regulation of calumenin, an endoplasmic reticulum chaperone that plays a central role in the regulation of gamma-carboxylation of vitamin-K dependent proteins, such as Matrix Gla-Proteins (MGP). Calumenin acts by inhibiting VKOR (vitamin K 2,3-epoxide reductase), the enzyme that produces reduced vitamin K, a required cofactor for gamma-carboxylation of vitamin K-dependent proteins. Furthermore, VKOR has been shown to harbor a thioredoxin-like CXXC center involved in reduction of vitamin K1 2,3-epoxide (Vit.K>O). This reduction is linked to the dithiol-dependent oxidative folding of proteins in the endoplasmic reticulum by protein disulfide isomerase (PDI). In beta-thal-PXE+ fibroblasts, as in PXE fibroblasts, there is a significant down-regulation of PDI, consistently with the impairment of the complex system responsible for the correct carboxylation of MPG.

It has been recently suggested that in PXE elastic fiber mineralization and reduced MGP-carboxylation are caused by the absence of a basolateral plasma membrane transporter (i.e. MRP6) in the liver, thus causing an insufficient amount of circulating vitamin K for the periphery to completely cover carboxylation requirements [7]. Present data get new light on the role of vitamin K and of MGP carboxylation in the pathogenesis of ectopic calcifications, since even in beta-thal-PXE+ patients ABCC6 expression is normal and therefore alterations are not related to a reduced availability of vitamin-K from the liver, but could be related to impaired ability of its recycling, possibly due to altered endoplasmic reticulum environment as a consequence of increased oxidative stress leading to cell metabolic alterations similar, although not necessarily identical, to those in inherited PXE.

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MOLECULAR GEOMETRY OF COLLAGEN FIBRIL ANCHORAGES TO DECORAN, BIGLYCAN AND FIBROMODULIN IN EXTRACELLULAR MATRICES (ECMS).

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INTRODUCTION. Decoran:collagen fibril interactions are of great importance in animal biology, underpinning the organisation of connective tissue ECMs, thereby maintaining the shape of the organism. Decoran, which carries dermochondan sulphate chains, binds to specific sites on collagen fibrils located in the d & e bands in all ECMs so far examined [1]). Keratan sulphate-bearing analogues bind in the a & c bands in corneas and probably in cartilages. Amino-acid sequences (GDRGE etc) found in these bands were suggested to be part of the binding sites [2]. Biochemical evidence and in vitro models using purified decoran and collagen support these findings.

Two modes of interaction were proposed based on horseshoe-shaped images of rotary-shadowed decoran [2]. The decoran protein (decoron) could accommodate a collagen molecule in its jaws, but in the preferred alternative it bridged neighbouring collagen molecules, engaging similar sites on each. Charge motifs (- + 0 -) in collagen binding sites complemented those (+ - 0 +, RELK, RELH) in decoron [2]. One group chose the first alternative and computer-modelled binding of a piece of collagen with a simulated decoron structure based on ribonuclease inhibitor. There was no link between the observed collagen binding sites (above) and the collagen piece while the computed decoron structure differed considerably from the structure later derived from X-ray scattering [3]. The evidence-based structure [2] was a 2-dimensional construct and the crucial question is, can it exist in 3 dimensions?

RESULTS AND DISCUSSION. The X-ray data show that the proposed binding residues RELK (101-104) and RELH project from the leucine rich-repeat core with little hindrance to interactions involving them. It also suggests that the sequence SYRI[4] may cooperate with the sequences originally proposed [2] to give multi-pronged binding. Courtauld models show that the decoron and collagen charge motifs fit very well together. Computer simulation using atomic coordinates from crystallographic investigations [5] shows that collagen fibrils can accommodate these structures.

Biglycon and fibromodulon possess similar putative binding sequences to those in decoron (TABLE) and their proposed binding sites on the fibril also have similar complementary sequences. Lumicon does not contain similarly placed charge motifs, suggesting that it is not bound to the fibril analogously to the other three proteoglycans.

TABLE

Decoron	KLER(LYL)	(L)RELH(L)	N? C
	101	243	
Biglycon	KLQK	(L)RELH(L)	N? C
Fibromodulon	HLER(LYL)	LELD	N? C

'101' collagen-binding motives correspond exactly in primary sequences aligned for maximum homology. The '243' motive in fibromodulon is not a typical motive but an exact copy (RELH) is present only 24 residues, instead of ~122 in the other proteoglycans, towards the C terminal. Lumicon has no similar motives in corresponding positions

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GENE EXPRESSION OF *XENOPUS LAEVIS* VERSICAN ISOFORMS DURING EMBRYONAL DEVELOPMENT

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Versican, a member of the hyalectan family proteoglycans, is a primary component of the vertebrate embryonic extracellular matrix, where it is supposed to play crucial roles during development. Its complex strutture provides the basis for its multiple and sometime contrasting effects on cellular functions, such as cell adhesion, cell migration, cell proliferation and apoptosis, as well as on morphogenesis (1). In the mouse, deletion of versican gene leads to severe cardiovascular malformations and embryonic lethality.

In a previous study (2) the full-lengh versican cDNA was identified in *Xenopus laevis* embryos (Stage 30), as a coding sequence of 11.559 bp, codifying a putative core protein of 3852 amino acids. The comparison of the amino acid sequence with that of other vertebrate orthologues shows the typical structure of the versican core protein, composed of a G1 hyaluronan-binding domain flanked by the two well-described GAG attachment regions, GAG-alpha and GAG-beta, and the multimodular G3 domain embodying an EGF repeat, a lectin-like module and a CRP repeat. G1 and G3 domains of Xversican show high homology with the human versican, while the homology drastically decreases in the GAG-alpha and GAG-beta domains. In addition to the full-lengh V0 isoform we identified (2) five spliced variants corresponding to the isoforms V1a and V1b, both lacking most putative GAG-alpha binding sites, V2a and V2b, lacking the majority of Gly-Ser-Gly sequence of GAG-beta, and V3, lacking all GAG binding sites. In situ hybridation of V0 isoform on embryos at different stages of development was also performed, to show that versican is early and dynamically expressed during Xenopus embryogenesis (2). It is mainly detected in the final sites of arrest of neural crest cells (NCC), in a sub-population of trunk NCC migrating into the fin and in tissue flanking the trunk NCC, correlating with their patterns.
In the present work we have undertaken an analysis of the expression pattern of Xversican spliced isoforms during development, by focusing on developmental stages 5, 18, 27 and 38, and have further identified an additional splice variant, named V1c, with a lenght of 8904 bp - 2967 amino acids. We have also examined the product (parental isoform, V0-2) of a second Xversican gene, having a coding sequence of 11517 bp and codifying for a putative core protein of 3856 amino acids.

The gene expression evaluation was carried out by RT-PCR real-time using TaqMan chemistry, and results are expressed as negative or positive multiples of the value of the V0 isoform of gene 1 (V0-1) at developmental stage 5, considered arbitrarily equal to one. At this stage, only the V3 was expressed in a similar amount as V0. Expression of V0 decreases during development, in a similar way for the products of both gene 1 and 2, although the latter one was found much less expressed than the former. On the contrary, the expression of isoform V3, lacking the GAG binding regions, increases with development.

The results are consistent with observations made in other species, suggesting that V0 represents a typical embryonic isoform, while highlighting a completely new pattern of expression of the V3 isoforms not previously identified in other vertebrate embryos as a main isoform. It is noteworthy that only these two variants are commonly expressed as positive multiple of the reference V0 at stage 5. The others variants are less expressed, and they sometime show further decrease with development, as V1c and V2b, or roughly constant expression (V1b), or detectable expression only at final stage (V1a and V2a).

The study shows that two different versican genes exist in *Xenopus* embryos, although only one is sufficiently expressed, due to a different sequence or methylation profile of the promoter. The two gene products are very similar and the small differences that were detected may involve glycosylation at the GAG-beta binding region which could be of significance for the control of embryonic cell movement. This idea is supported by the observation that the fully glycosylated V0 variant decreases with development, while the unglycanated V3 variant increases.

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PROTEOMIC CHARACTERIZATION OF CULTURED AGED HUMAN DERMAL FIBROBLAST.

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Aging is commonly defined as the accumulation of diverse deleterious changes occurring in cells and tissues, which could be responsible for impaired functions of affected organs and for the age-dependent increased incidence of several diseases, i.e. risk of cancer occurrence, ischemic diseases like acute myocardial infarction and stroke, metabolic disorders as diabetes, vascular alterations leading to atherosclerosis and/or impaired wound healing [1]. Moreover, it is known that environmental factors may influence cell behaviour, and that the response of genes involved in the defence against oxidative stress and transcriptional regulation is severely impaired in old fibroblasts [2,3]. Fibroblasts are important stromal cells that synthesize the structural components of the extracellular matrix, and migrate within the stroma in order to interact with other cells and with the extracellular milieu according to different stimuli [4,5] . Previous studies demonstrated that that human fibroblasts cultured in-vitro from young, adult and old donors retained, in vitro, at least some of the characteristics they exhibit in-vivo not only regarding proliferative capabilities but also as far as metabolic and degradative pathways as well as the ability to cope with endogenous and/or exogenous stresses [6]. However, the great majority of proteins did not appear to significantly change their expression when cultured in-vitro, consistently with the hypothesis that senescence may be the result of both genetic and epigenetic factors [3,4].

Aim of the present study was to grow fibroblasts from young (< 15 years) and old (> 80 years) donors and to compare protein expression at low (6-8) and high (28-30) CPD (cumulative population doublings).

Results demonstrated that parameters of oxidative stress, revealed by the DH_2 and H_2-DCFDA fluorescent probes, are significantly increased in fibroblasts from old donors, already at low CPD, and that values further increase with in-vitro senescence. Furthermore, age-dependent accumulation of ROS products is associated, for instance, to down-regulation of thioredoxin, which is particularly evident in fibroblasts from old donors already at low CPD, indicating that redox homeostasis is permanently altered in dermal fibroblasts depending from in-vitro aging but also from donor's age. By contrast, well known senescence markers as beta-galactosidase and caveolin-1 exhibited very similar values when fibroblasts from young and aged donors were placed in culture, but showed a significant increase with in-vitro aging (CPD 8 vs CPD 30). Similarly, other proteins present in the endoplasmic reticulum (ER), as protein disulfide isomerase, calreticulin and 78 kDa glucose regulated protein, were significantly down-regulated only in in-vitro aged fibroblasts confirming that down-regulation of ER luminal proteins in in-vitro aged fibroblasts is consistent with in-vivo age-related modifications of protein processing and folding, calcium homeostasis and ER-associated apoptotic pathways. Furthermore, longevity seems to show correlation to the cellular level of chaperone expression [7], reflecting the ability of the housekeeping molecular chaperones to stabilize the normal phenotype. Among these chaperone molecules, Hsp60 appeared to be significantly down-regulated only in in-vitro aged fibroblasts, independently from donor's age, further supporting the hypothesis that the attenuated response to stress, characteristic of senescence, may have consequences on the ability to aging organisms to cope with the increased incidence diseases. Over the last decades it has become evident that the cellular-physiological functions of the organism do not all decline at the same rate with age, and that the faster decline concerns the elastic functions of the organism as a consequence, for instance, of the extremely low turnover of the elastic components and to its susceptibility to endogenous as well as exogenous noxae [8]. Evaluation of matrix gene expression, mainly focusing on elastic fiber–related components, demonstrated that elastin mRNA expression was moderately affected by donor's age or by in-vitro senescence, whereas significant changes were noted for fibulin-5 mRNA expression that appeared significantly reduced in fibroblasts from aged donors and in in-vitro aged cells. Data underline the importance of fibulin-5 as a good marker of ageing skin and as a fundamental modulator of elastic fiber assembly.

In conclusion, these data clearly demonstrate that fibroblasts from aged donors could represent a good model for investigating the relationships between senescence and oxidative stress, however, the great majority of parameters are modified only in in-vitro aged fibroblasts, supporting once more that cells at increasing CPD could be considered a more reliable model of senescence.

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FIBROBLASTS ENHANCE PROLIFERATION AND INVASION OF BREAST CANCER CELLS (8701-BC)

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The influence of tissue microenvironment on tumor biology has been predicted more than 100 years ago, since Paget's seed and soil' hypothesis. The 'soil', or the microenvironment for tumor cells, is composed of numerous different types of cells (including fibroblasts, myofibroblasts, myoepithelial cells, endothelial cells, macrophages) as well as ECM molecules [1, 2, 3]. Although epithelial and mesenchymal interactions have been widely acknowledged as crucial effectors for organ development, growth, and differentiation, in the past decades the major focus of cancer research has been on the tumor cell itself. It is becoming increasingly apparent that the tumor microenvironment plays a critical role in human breast cancer onset and progression. Epithelial-mesenchymal interactions often involve secreted factors, contact factors, or growth factors embedded within the extracellular matrix. Therefore, the extracellular environment of primary tumors may be depicted as a complex microecosystem, in which the neoplastic cells, the extracellular matrix, mesenchymal cells, blood and lymphatic vessel, and a variety of active factors play a dynamic role in cancer progression.

Among host cells, fibroblasts are likely to be the most representative ones, since they are involved in deposition and remodelling of the ECM and responsible for the secretion of many active factors. The aim of this study was to investigate the effects of fibroblast's stimulation on proliferation and invasiveness of breast cancer cell line cells 8701-BC. Growth rate, as well as migration and invasion assays were performed utilizing fibroblast conditioned medium on the 8701-BC cell culture. Parellel anlyses were performed by co-culture experiments using transwell chambers and microporous membranes that allow the passage of soluble moleules and retain cells. Our results indicate that 8701-BC are responsive to fibroblasts stimulation, showing an increase of proliferation rate enhancement of migration and invasion. These effects were associated with a significant increase of cyclin B and c-Myc protein expression (figures 1A and 1B). Cyclin B is the large protein subunit of mitosis promoting factor and represents one of the major cell cycle controllers. Abnormalities of cell cycle regulators, including cyclins and cyclin dependent kinases, have been reported in various malignant tumors. Its augmented expression under fibroblast influences is in agrement with the observed effect on cell proliferation. Also coherent with these data is the increment of oncoprotein c-Myc. MYC is indeed the most commonly amplified oncogene in human breast cancer, involved in the network that regulates a large number of cellular functions. Deregulation of MYC may cause uncontrolled cell proliferation, autonomous cell growth, genetic instability, escape from immune system and cellular immortality [4]. Finally, we assayed the activity levels of matrix metalloproteinase-9 and -2 (MMP-9 and MMP-2), which are known to play a critical role in tumor invasion and metastasis and therefore regarded as possible pharcological target [5,6]. As shown in figure 1C, cells stimulated by fibroblasts display about twofold increase of MMP-2 activity.

Taken together our data indicate that factors derived from human normal fibroblasts can stimulate further the potentiality of 8701-BC cells, which are known to be key features for tumor progression.

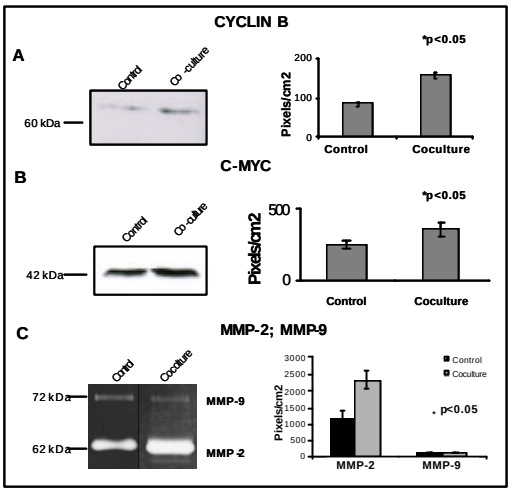

Figure 1: Panel A and B show the western blot analysis of cyclin B and eMyc expression in 8701-BC control cells and co-cultered with fibroblasts. Panel C displays the activity level of pro-MMP-9 and -2 detected in breast cancer cells conditioned medium.

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STUDIES ON THE POST-TRANSLATIONAL MODIFICATION OF HYALURONAN SYNTHASES (HAS)

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Hyaluronan is a linear glycosaminoglycan of high molecular weight composed of the repeating disaccharide unit *N*-acetyl-D-glucosamine-ß(1? 4)-D-glucuronic acid-ß(1? 3). Hyaluronan is a component of the connective tissue and is present in the extracellular matrix of most vertebrate tissues. It plays an important role in several cellular events, such as proliferation, migration and adhesion, whereas it is implicated in many morphogenetic processes during vertebrate embryogenesis [1]. Perturbations in hyaluronan levels can lead to dramatic changes in tissue homeostasis. It has been demonstrated that a close correlation between elevated hyaluronan synthesis and tumor progression and/or inflammation.

Hyaluronan is synthesized by three membrane integrated hyaluronan synthases (HAS1, 2 and 3). The three HAS enzymes not only synthesize HA but they also have a large number of functions required for the overall polymerization of HA. For example they act as binding sites for each of the two different sugar nucleotide precursors (UDP-GlcNAc and UDP-GlcA), they have two different glycosyltransferase activities, and they act as one or more binding sites that anchor the growing HA polymer to the enzyme, and catalyze a ratchet-like transfer reaction that moves the growing polymer one sugar at time. The expression pattern of each Has isoform differs between normal cells and their transformed counterparts as well as between different tumor types suggesting different functional roles [2].

Ubiquitination, a post translational modification, is an important mechanism in many biological processes, including cell cycle progression, transcription and signal transduction. Ubiquitin modifications involve mono-, multi- and polyubiquitination driving the protein to different biological processes. Linkages in polyubiquitin chain can be either at Lys48 of ubiquitin that is mainly used for targeting to the proteasomes, or Lys63 that seems to play important role in DNA damage tolerance, inflammatory response, the endocytic pathway and ribosomal protein synthesis [3].

To investigate whether HAS2 has occurred oligomerization or ubiquitination, we transfected COS-1 cells with plasmids containing Flag- or 6myc-tagged Has2. The tag-HAS2 transfected in COS-1 was immunopricipitated from cell lysates using antibodies against the tag, and subjected to SDS-PAGE, followed by immunoblotting or silver staining. The analysis resulted in bands with molecular masses corresponding to HAS2 monomers and oligomers. That the bands corresponded to HAS isoforms, is confirmed by peptide mass finger printing using MALDI-TOF-MS. Western blot analysis using antibodies against ubiquitin or polyubiquitin chain showed that HAS2 is both mono-and polyubiquitinated. To elucidate whether polyubiquitin chain is Lys48- or Lys63-linked, hemoglutinin-tagged ubiquitin K48R and K63R mutants were co-transfected with Flag-Has2 in COS-1 cells, followed by immunoprecipitation and western blotting. Polyubiquitination of HAS2 was observed with both mutants of ubiquitin but with a stronger signal of the Lys48-linked chain (i.e. K63R mutant). This observation suggests that the main role of ubiquitination is to lead the HAS2 to proteasomal degradation. The last data was confirmed by treating cells with the proteasomal inhibitor MG132, showing accumulation of polyubiquitinated HAS2. Currently, we sought to explore the role of monouniquitination in the HAS2 activity.

To sum up, in this work we investigated that HAS2 is post-translational modified by oligomerization and ubiquitination. A Lys48-linked polyubiquitination of HAS2 leads the protein to proteasomal degradation, whereas preliminary results showed that monoubiquitination is involved in the activity of HAS2.

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HUMAN SERUM ALBUMIN SULFHYDRYL GROUPS OXIDATION IN PLASMA AND ATHEROSCLEROTIC PLAQUE EXTRACTS

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Human serum albumin (HSA) is the most abundant multifunctional plasma protein. It accounts for both transport and antioxidant functions, such as ROS/RNS scavenging, extracellular redox balance, redox active transition metal ion binding. Furthermore, albumin is a strong in vitro inhibitor of apoptosis. Many of these functions are related to the high reactivity of the redox active free Cys^{34} residue, due to its low pKa of approximately 7.0 compared to 8.5 and 8.9 for cysteine and glutathione, respectively. This residue accounts for 80% (500 μmol/L) of total thiols in plasma. It is present primarily in the reduced form (mercaptalbumin), although about 30-40% could be variably both reversibly oxidized (non-mercaptalbumin), as mixed disulfide with low molecular weigth thiols (LMW-thiols), S-nitroso Cys, sulfenic acid, and irreversibly oxidized, as sulfinic or sulfonic acid [1,2]. It has been described that the oxidation state of Cys^{34} is related to several physio-pathological conditions. The formation of mixed disulfides between LMW-thiols (cysteine, cysteinylglycine, homocysteine, glutathione and glutamylcysteine) and protein-SH to form thiolated proteins is known to occur after oxidative stress and has been recently suggested as a possible means of redox regulation of protein functions [3]. We have recently evidenced, by means of a proteomic approach on endarterectomy specimens from human carotid arteries, that the majority of extracted proteins was of plasma origin (about 70% of total proteins), being albumin the most represented [4].

The aim of this work was to evaluate if the pro-oxidant environment present in atherosclerotic plaque could oxidatively modify the infiltered albumin.

We evaluated albumin-Cys^{34} total oxidation by non-reducing SDS-PAGE of fluorescein-5-maleimide adducts. To estimate HSA total protein content and the degree of Cys^{34} residue oxidation in both plasma and plaque extracts we constructed two calibration curves with commercially standard BSA. Intra- and inter-assay CVs were estimated and purity of resolved HSA was proved by diagonal electrophoresis. Values are reported as fluorescent band intensity / μgHSA. Correlation coefficients of 0.999 for both calibration curves and CV values of 2.48% and 4.40%, for intra- and inter-assays, respectively, were obtained. Moreover, we evaluated both the level and the pattern of human serum albumin S-thiolation by capillary zonal electrophoresis with a laser induced fluorescence detection system (CZE-LIF).

Analysis of Cys^{34} total oxidation evidenced deep differences between plasma samples and the corresponding plaque extracts $p < 0.001$ (25665.3 ± 6865.7 vs 9171.9 ± 5184.7), indicating that circulating HSA, once filtered in arterial wall, is subjected to Cys^{34} oxidative modifications, probably due to the strong pro-oxidant environment. Moreover, filtered albumin showed a different pattern of bound LMW-thiols.

The relevance of these oxidative modifications in the patho-physiology of atherosclerotic plaques is not completely known. Further studies are needed to evaluate the other types of Cys^{34} modifications (HSA-SNO, HSA-SOH, HSA-SO_2H, HSA-SO_3H) and their implications in the development of the disease.

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DIRECT GAG-COLLAGEN INTERACTION: MORE QUESTIONS THAN ANSWERS

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The extracellular matrix (ECM) macromolecules form a network of complex interactions mainly determined by hydrophilic/hydrophobic and electrostatic forces. These weak bonds are easily altered by the treatments required by some ultrastructural techniques, a condition which results in frequent inconsistencies in the scientific literature. Moreover, because of the structural similarity among small leucine-rich proteoglycans (SLRPs), any missing or defective molecular species *in vivo* is likely to be compensated by the overexpression of some other type, limiting the usefulness of molecular biology techniques and adding to the general uncertainty.

In order to gain fresh data on the collagen-proteoglycan interaction we reconstituted collagen fibrils in the presence of highly-purified proteoglycans (PGs) or glycosaminoglycans (GAGs) under controlled conditions in a very simple cell-free environment. Our engineered ECMs were observed by atomic force microscopy (AFM) and high-resolution scanning electron microscopy (SEM) after the simplest possible pre-treatment. The first, recently published results (1, 2) revealed a few new data but left behind a far greater number of open questions.

Acid-soluble type I collagen (ASC I) by itself forms a three-dimensional, isotropic network of slender fibrils whose diameter depends on the initial concentration of collagen. A concentration of 0.5 mg/ml gives an average diameter of 50.6 ± 8.5 nm (n=50). The fibrils show a normal D-banding but have an extreme tendency to merge laterally into thick, irregular super-fibrils or into a continuous web.

The presence of decorin during fibrillogenesis causes the fibrils to grow to an average diameter of 55.6±8.8 nm, n=50 (significantly larger than controls, $p<0.01$). Lateral fusions are completely abolished, and the fibrils themselves are gathered into compact, tight bundles reminiscent of the fibres found in connective tissues. Pre-treatment with Cupromeronic Blue (CB) causes the GAG/CB complexes to become clearly visible, most of them forming regular, ladder-like interfibrillar bridges. The decorin core has a similar effect on the fibrils diameter (57.1 ± 9.1 nm, n=50, not significantly different from the effect of intact decorin) and on the lateral fusion.

The addition of the purified side chain of decorin, which essentially a is copolymer of chondroitin 6-sulphate and dermatan sulphate, caused the fibril diameter to drop to 26.5±12.7 nm, n=50 ($p<0.01$). Pre-treatment with Cupromeronic Blue reveals belt-like loops running orthogonal to the fibril axis and encircling two or more fibrils in an obvious correlation with the collagen D-period, indicating that GAGs are capable of a specific interaction with collagen fibrils even in absence of any core protein.

Similar, if slightly reduced effects, are observed after addition of chondroitin-sulphate B (totally iduronic acid-4-sulfo-hexosamine) and of chondroitin-sulphate C (mostly glucuronic acid-6-sulfo-hexosamine). The fibrils diameters are respectively of 36.3±5.7 nm and 39.7±4.9 nm, both significantly smaller than control fibrils ($p<0.01$). By contrast, chondroitin-sulphate A (mostly glucuronic acid-4-sulfo-hexosamine) had no visible interaction with the collagen fibrils, presumably because of the steric hindrance of its sulphate groups.

The functional significance of these data are still being debated. It is generally agreed that SLRPs contribute to the functional properties of tensile tissues by providing interfibrillar mechanical coupling (see, for instance, 3). Yet the latest measured values for GAG-to-GAG adhesion strength (4) are inconsistent with the biomechanical properties of tendons and, in particular, with the estimated critical length of collagen fibrils, falling short of approx. three orders of magnitude. In tendons, however, collagen fibrils are tightly packed in close contact and often deformed by the reciprocal pressure, so that their interfibrillar GAGs often contact the fibril surface with their whole length. These GAGs may therefore act as an interfibrillar glue, providing an important source of extra adhesion. An important hint in this direction comes from the observation that the addition of chondroitin-6-sulphate to a collagen matrix causes a marked increase of its mechanical properties, comparable to the effect of chemical cross-linking (5). Research is still under way.

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OSTEOGENESIS IMPERFECTA: USE OF THE BRTLIV MURINE MODEL TO BETTER UNDERSTAND THE MOLECULAR BASIS OF OI AND TO DEVELOP NEW THERAPEUTIC APPROACHES

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Osteogenesis Imperfecta (OI) is a genetic dominant bone disorder characterized by skeletal fragility and deformity and caused by mutations in the type I collagen genes. Recently recessive forms of OI have been recently described due to mutations in genes related to collagen post translational modifications. The OI clinical outcome is extremely variable ranging from very mild to lethal forms.

BrtlIV is a knock-in murine model for the classical dominant OI, which carries a typical glycine substitution (Gly349Cys), the dominant transmission and whose phenotype models the moderately severe human OI (1). BrtlIV shows a moderate/severe or a lethal OI outcome reproducing the phenotypic variability reported for human patients (2). The availability of a mouse model represents a valid tool both to investigate the molecular basis and to develop new therapies for this disease.

To understand the molecular basis of the phenotypic variability, we investigated bone mRNA expression by microarray and bone protein profile by 2-DE and mass spectrometry in the OI murine model BrtlIV.

In particular we found an increase in lethal BrtlIV of Gadd153 and a lower expression of the αBcrystallin that indicated an effect on the phenotypic outcome of the intracellular machinery encountering retention of the mutant collagen (3). Using in vitro cultured calvarial osteoblasts from newborn lethal and non lethal BrtlIV mice we demonstrated a slower proliferation and a delayed mineralization in cells from mutant mice with lethal outcome. We also determined in those cells by RT-PCR an increase in Gadd153, Bim and Cepbα and a decrease in Cebpβ expression suggesting the activation of an apoptotic pathway.

Since OI is a genetic disorder no definitive cure is at the present available, but a lesson from nature tells us that mosaic carriers of collagen mutations, some of the parents of OI patients, have very mild or absent clinical outcome. Cellular therapy is the treatment that mimics the mosaic situation, thus could be an effective therapy for OI. Based on this consideration we developed a cellular transplantation treatment in BrtlIV. We employed *in utero* bone marrow transplantation both to avoid marrow ablation and because OI is an inborn disorder. The bone marrow cells were isolated from long bones of eGFP-CD1 mice and injected into E13.5-14.5 BrtlIV and WT embryos. Mice were analyzed at 2 m, the age corresponding to the severest BrtlIV bone phenotype compared to WT (4). Engraftment with a characteristic patchy distribution was detected in various tissues at sacrifice by inverted fluorescence microscopy. Confocal microscopy was used to directly quantify the engraftment in long bone diaphysis. The percentage of donor cells was determined by FACS, in both bone marrow and spleen and by Real Time PCR in different tissues. PQCT of the distal femoral methaphysis revealed increased total bone and trabecular density and pQCT of the distal tibia methaphysis revealed increased total bone and cortical density in treated versus untreated mutant mice. Micro CT analysis of Brtl mid-shaft femur detected improvement in Total Mineral Content, Cortical Thickness and Cortical Area. Biomechanical studies showed an improvement in yield load, ultimate load and stiffness. The predicted ultimate strength and the predicted elastic modulus were increased in BrtlIV treated with respect the WT. Evaluation of the collagen composition of the bone indicated that about 20% of the collagen was synthesized from normal donor cells. Ultimately to evaluate the cellular therapy effects is necessary to have a better knowledge of how the endogenous stem cells behave in the natural bone marrow environment. We investigated in BrtlIV the MSCs proliferation and differentiation toward adipocytes and osteoblasts. MSCs proliferation was statistically higher in Brtl mice than in WT. No difference was detected in the CFU-F number, but the ability of mutant cells to differentiate to adipocytes was greater and their ability to differentiate toward mineralizing osteoblasts was less than in WT.

Based on our *in utero* treatment the stem cell transplantation seems promising to cure the disease, but the abnormal endogenous MSCs proliferation and differentiation found in our OI model suggest that further analysis need to be performed to optimize and better evaluate the cellular therapy approach.

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INFLAMMATION AND RESOLUTION IN CARTILAGE: INVOLVEMENT OF COX-2

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We previously demonstrated that, in the MC615 cartilage cell line, the p38/NF-kB pathway is activated both during differentiation and in response to an inflammatory stimulus. In both cases, the p38/NF-kB pathway activation leads to the expression of the lipocalin SIP24 and of COX-2. Given the fact that, in the same cells, the COX-2 expression is sustained during the inflammation resolution, at the same time that the SIP24 expression is suppressed, in the present study we tested the hypothesis that COX-2 products play a role in SIP24 repression. Results are: 1) 15-deoxy-delta 12,14-prostaglandin J_2, but not PGE_2: i) inhibits the expression of SIP24 in the inflammatory phase and induces COX-2 synthesis; ii) represses NF-kB activation induced by LPS; iii) represses the synthesis of microsomal PGE Synthase-1 induced by LPS. 2) PPARγ and PPARα are present in MC615 cells in both proliferating and hyperconfluent cultures. 3) PPARγ ligand GW7845, but not PPARα ligand GW7647: i) represses the expression of SIP24 induced by LPS; ii) induces COX-2 expression. 4) p38 is involved in the PPARγ mediated induction of COX-2. In fact 15-deoxy-delta 12,14-prostaglandin J_2 activates p38 and the cell pretreatment with the p38 specific inhibitor SB203580 represses the expression of COX-2 induced by both the 15-deoxy-delta12,14-prostaglandin J_2 and the PPARγ ligand GW7845.

Taken together, our results suggest that, during the resolution of inflammation, COX-2 represses the acute phase protein SIP24 and restores physiological conditions, possibly through a pathway involving PPARγ.

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ABCC6 MUTATIONS IN ITALIAN PXE PATIENTS: AN UPDATE DESCRIBING TWENTY-TWO NOVEL MUTATIONS.

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Pseudoxanthoma Elasticum (PXE) is a rare heritable disorder inducing mineralization of elastic fibers. The disease is caused by mutations in the ABCC6 gene that encodes for a membrane transporter, mainly expressed in liver, and whose physiological role is still unknown. At present more than two hundred different mutations were found throughout the ABCC6 gene [1,2]. Unfortunately, PXE clinical manifestations are characterized by various degrees of severity, even in subjects bearing the same mutations, and within the same family, making difficult any genotype-phenotype correlation [3]. Furthermore, the heterogeneity of clinical manifestations allows to hypothesize that other genes/polymorphysms or environmental factors may take part in the phenotypic expression of the disease. For instance, we have recently demonstrated that different parameters of the redox status are significantly modified in the circulation of PXE patients compared to control subjects of comparable age and that the oxidant/antioxidant ratio (OX/AntiOX ratio) was significantly increased in patients and related to the extent of clinical manifestations in patients with identical mutations [4]. Data will be presented on new mutations identified in Italian PXE patients, with the aim to increase the knowledge on the expanding spectrum of ABCC6 mutations.

Blood and skin samples were taken from 174 PXE patients all belonging from Italy. DNA was analysed for mutations by amplification and direct sequencing. Skin biopsies were analyzed by light and electron microscopy. Clinical data were carefully collected from all patients. Genomic DNA samples from 50 unaffected and unrelated individuals were used as controls.

PXE patients included in the present study belonged to 139 families: 98 patients were from Nord Italy, 40 from the Centre, 30 from Sud and 6 from Sicily and Sardinia. Among the mutations that we have demonstrated, 23 were already described in a previous report [5], 27 were reported by other Authors [1], whereas 22 appeared to be new mutations, that were never described.

In particular, these novel mutations were: c.123dupC, p.M42HfsX59 (exon 2); c.196dupT, p.S66FfsX35 (exon 2); c.557delT, p.L186RfsX46 (exon 5); c.913C>T, p.Q305X (exon 8); c.940G>A, p.G314R (exon 8); c.989delA, p.K330SfsX26 (exon 8); c.1220G>T, p.G407V (exon 8); c.1284C>G, p.N428K (exon 10); c.1308G>A, p.W436X (exon 10); c.1779+1G>C (intron 13); c.2266G>A, p.G756S (exon 18); c.2728_2746 dup19, p.W9 (exon 21); c.2678C>A, p.S893 (exon 24); c.3507-3C>T (intron 24); c.3542G>A, p.G1181D (exon 25); c.3707T>C, p.M1236T (exon 26); c.3712G>T, p.D1238Y (exon 26); c.3774dupC, p.W1259LfsX19 (exon 27); c.3871delG, p.A1291QfsX68 (exon 27); c.4041G>A (exon 28); c4070G>C, p.R1357P (exon 29); c.4208+1G>A (intron 29). Of these mutations eight were missense, three nonsense, six frame-shift, one small insertion and four putative splice site. Moreover, two mutations were located in the extracellular, nine in the transmembrane and eleven in the intracellular domains. The mutation detection rate was 92,5%.

In conclusion, these data further contribute to establish a detailed map of ABCC6 mutations in Italian PXE patients. Moreover, comparison of clinical data, histological findings and ABCC6 mutations further sustain the hypothesis that other factors and/or eventually other gene(s) may contribute to the occurrence and to the severity of the disease. We have already demonstrated that heterozygous PXE carriers exhibited several alterations very similar, although less severe, to those typical of PXE patients [6] indicating that heterozygosity for ABCC6 gene expression is sufficient to cause elastic fibre calcification and connective tissue abnormalities, possibly in combination with other gene mutations as recently demonstrated in a family with Pseudoxanthoma elasticum-like phenotype [7].

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CORRELATION BETWEEN APOE POLYMORPHISM AND SEVERITY OF CARDIOVASCULAR MANIFESTATIONS IN PSEUDOXANTHOMA ELASTICUM (PXE).

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Pseudoxanthoma elasticum is a human genetic disorder characterised by progressive calcification of elastic fibers (1) in the skin, the Bruch's membrane of the retina and the cardiovascular system. It is due to mutations in the ABCC6 gene (2) coding for a membrane transporter whose physiological function is unknown. The pathogenesis of elastin calcification is still under investigation. There are data suggesting that PXE is a rather complex metabolic disorder where alterations in membrane transporter MRP6 and circulating factors perturb elastic fiber formation and stability (3,4). Vascular alterations are particularly relevant in PXE and progress up to occlusions, hemorrhages and legal cecity.

We have shown that PXE fibroblasts in vitro suffer from a chronic oxidative stress (5) and are not able to produce fully carboxylated Matrix Gla Protein (MGP), an inhibitor of calcium precipitation (6). We proposed that immature MGP may be responsible for elastic fiber calcification in PXE (6). As carboxylase is a vitamin-K-dependent enzyme, all conditions decreasing vitamin K availability would lead to impaired MGP ?-carboxylation and favour elastic fiber calcification (6). It is known that dietary vitamin K is carried to liver and to peripheral tissues by the same lipoprotein fractions involved in ApoE transport and metabolism (7) and that the ApoE e4 isoform is the less efficient in favouring the utilization of vitamin K introduced with the diet (8). Therefore, ApoE polymorphism may affect ?-carboxylase activity and could be relevant in PXE clinical manifestations.

Apo E polymorphisms were identified in 104 PXE patients. The frequency of the ApoE alleles was similar to that in the normal population, being alleles e3 84%, e4 9% and e2 7%. The genotype distribution was 69% e3e3; 16% e3e4; 12% e2e3; 3% e2e4. PXE patients with the allele e4 had higher circulating levels of cholesterol ($p<0.004$) and LDL ($p<0.002$) compared with patients with genotype e3e3. Moreover, e4 allele was associated with higher levels of TG and lower levels of HDL compared with subjects e2e3 and e3e3. As expected, cholesterol, LDL and TG increased linearly with age of patients; by contrast, HDL remained constantt or tended to decrease with age, especially in e2e3 patients. Intermittent claudication, gastrointestinal haemorrhages and cerebral infarcts started to appear on the third-fourth decade of life and their frequency increased with age (Table I). Claudication affected about one third of patients in the third decade of life and 70% of patients over the age of fifty, whereas haemorrhages affected about one third of patients over sixty. When related to the ApoE genotype, intermittent claudication was present in 63%, 45% and 33% of patients with alleles e4, e3 and e2 respectively. Scores from 0 to 4 were assigned to the severity of clinical manifestations and were related to genotypes. There was not significant difference in the skin score among the ApoE genotypes; by contrast, the cardiovascular score increased significantly from genotype e2e3 to e3e3 and to e2e4/e3e4. In conclusion, similarly to normal subjects, in PXE patients, the allele e4 is associated with higher plasma levels of cholesterol, LDL and TG and with lower levels of HDL. In accordance, PXE patients with the allele e4 have more severe and earlier cardiovascular alterations compared to patients carrying the e2 and e3 alleles. This should be taken into account when evaluating cardiovascular risk in PXE.

Table I
PXE patients with vascular clinical manifestations.

	10-19yr (8)*	20-29yr (13)*	30-39yr (25)*	40-49yr (21)*	50-59yr (22)*	60-77 yr (15)*
Intermittent claudication	37%	15%	32%	57%	73%	67%
Hypertension			12%	10%	59%	67%
Gastro-intestinal haemorrhage			8%	24%	18%	33%
Infarct				14%	14%	27%

* In parenthesis the number of subjects

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NEW INSIGHTS INTO THE IMPLANT OSTEOINTEGRATION TECHNIQUES.

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Bone tissue response to the insertion of an implant triggers a series of events in the cells and peri-implant matrix culminating in the intimal apposition of bone tissue at the implant surface, a process known as "osteointegration". Many studies have focused on perfecting implants to enhance the biological fixation and shorten the healing period. In this connection, biomolecular investigations must be flanked by morphological analysis of the tissue hosting the implant. Routine studies usually include histological analysis (light microscopy, LM) and ultrastructural observations (scanning electron microscopy, SEM; transmission electron microscopy, TEM).
Each of these techniques provides information confined to certain morphological features of the tissue. LM yields information on the two-dimensional structure of bone and vascular tissue next to the implant, providing general indications on the features and organization of the bone matrix. LM will also provide a broad overview of the peri-implant area (Fig. 1a). SEM defines the three-dimensional structure of tissues surrounding the implant and offers more detail of the various biological structures involved in the osteointegration process thereby defining the surface cell and tissue morphology (Fig. 1b). Lastly, TEM discloses the macromolecular components of the matrix and its cells. The information provided by these different approaches is subsequently pooled to yield an in-depth picture of tissue morphology.
Different morphological investigations require specimens to be processed in totally different ways so that different specimens must be allotted for each technique. For this reason, the results obtained are often inaccurate and difficult to interpret.
This study aimed to devise a processing protocol for different types of morphological investigation on the same specimen to allow an exact comparison of results obtained by different investigation methods. The protocol developed from these experiments requires initial processing of the bone tissue containing the implant for LM examination. This is done using a routine method for hard tissues in which the specimen is embedded in a methylmethacrylate-based resin and subsequently cut with a sawing and grinding system to obtain 100 µm thick sections to be stained for LM observation. Sections are then deacrylated using a specific solvent and processed for SEM and TEM examination. Small fragments are taken from each deresinated section for subsequent TEM observation, whereas the remainder of the section is processed for SEM.
By allowing morphological analysis of the same specimen using different techniques, this processing protocol is particularly suited to studies in which tissue topography plays an important role in the interpretation of results, e.g.

studies disclosing different tissue structure in adjacent areas. This aspect allows the present deacrylation technique to find application in both normal and pathological anatomy investigations.

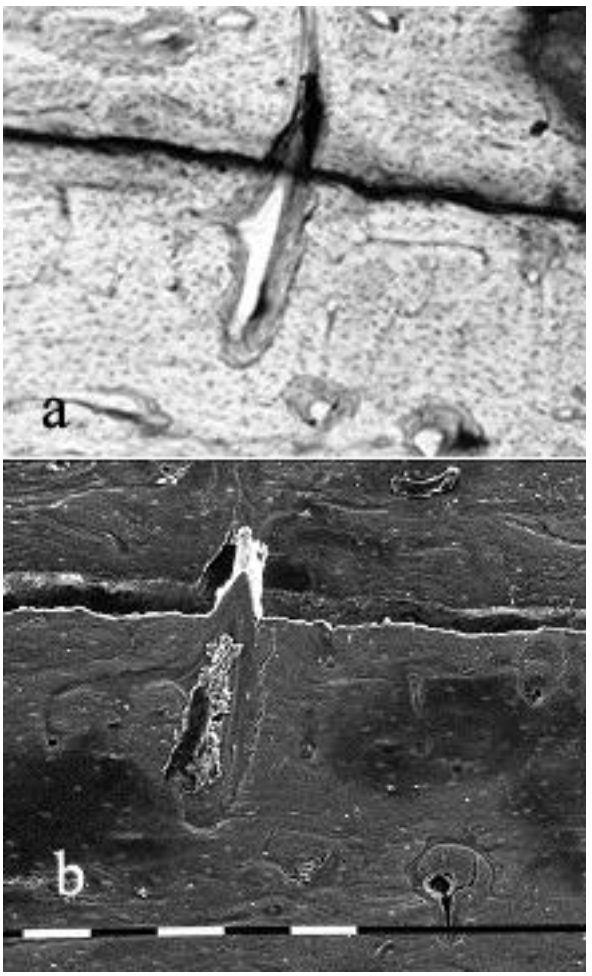

Fig.1: images showing a developing osteon included into mature host bone.a) light microscopy b)scanning electron microscopy.

STRATEGIES TO MODIFIED THE MATERIAL SURFACE WITH CALCIFIED MATRIX

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INTRODUCTION. One of the key challenges in reconstructive bone surgery is to provide living constructs that possess the ability to integrate in the surrounding tissue. Static culture environments suffer from limited diffusion and often result in inhomogeneous cell and extracellular matrix distribution. In order to overcome the drawbacks associated with static culture systems, several bioreactors have been designed to physically enhance the cell culture *in vitro* (1) with several stimuli.

MATHERIAL AND METHODS. $TiAl_4V$ 3D scaffolds (Lima-Lto, Villanova, Udine); electromagnetic bioreactor powered by a Biostim SPT pulse generator (PEMF) (Igea): magnetic field intensity, 2 mT; frequency, 75 Hz; human osteosarcoma cell line SAOS-2: from the American Type Culture Collection (HTB85, ATCC) cultured in McCoy's 5A: seeded 1×10^6 cells/ scaffold; standard culture: no PEMF stimulation, dynamic culture with PEMF stimulation; analysis perfomed: SEM, immunofluorescence, DNA content, RT-PCR , extraction of the extracellular matrix proteins and ELISA assay using a set rabbit polyclonal antibodies presented by L.W. Fisher (National Institutes of Health, National Instituteof Dental and Craniofacial Research, Craniofacial and Skeletal Diseases Branch).

RESULTS. We investigated the effects of electromagnetic stimulation on SAOS-2 human osteoblasts seeded onto a 3D titanium alloy scaffold. In comparison with control conditions, the electromagnetic stimulation caused increased surface coating with decorin, type I and III collagen, osteopontin, osteocalcin, osteonectin, and fibronectin [Tab.1]. The immunolocalization of the above proteins showed their co-localization in the cell-rich areas. RT-PCR analysis revealed the electromagnetically up-regulated transcription specific for the foregoing matrix proteins and for the growth factor TGF-? . Furthermore, the sample exposed to an electromagnetic bioreactor showed an higher cell proliferation as confirmed by the measurement of DNA content and SEM observations.

CONCLUSIONS. In order to overcome the total immunocompatibility with the patient, the use of mesenchymal stem cells (MSCs) could be promising. For this purpose, human bone marrow-derived MSCs (BMMSCs) were isolated from adult patient and their osteogenic potential was evaluated onto the same 3D titanium alloy scaffold in terms of cell adhesion, proliferation and differentiation in static conditions. We will be setting the same experiment with BMMSCs evaluating the effects of an electromagnetic stimulation. The use of an electromagnetic bioreactor aims at obtaining the surface modification of the 3D scaffolds interms of cell colonization and coating with calcified matrix; in this way the superficially modified biomaterial could be used, in clinical applications, as an implant for bone repair.

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Table 1

	Matrix protein coating after 24 days of culture in fg/(cell x scaffold)		
	Static culture	**Dynamic culture**	**Dynamic/Static**
Type-I collagen	48.1	196	4.07-fold
Type-III collagen	18.7	33.9	1.8-fold
Osteopontin	5.69	47	8.2-fold
Decorin	6.31	18.5	2-fold
Fibronectin	3.8	7.53	1.9-fold
Osteocalcin	3.53	11.2	3.2-fold
Osteonectin	3.05	7.9	2.6-fold

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HISTOMORPHOMETRIC EVALUATION OF IMPLANT DESIGN AS A FACTOR POSITIVELY INFLUENCING PERI-IMPLANT BONE RESPONSE. A PILOT STUDY IN DOG LOWER JAW.

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A fundamental role in obtaining long-term successful dental implants osseointegration is played by primary implant stability as the estabilishment of a direct bone-to-implant contact (BIC) with no interposition of connective tissue. Implant surface microtopography, implant material and design, original bone density have been widely studied as major factors positively influencing implant osseointegration. In our previous studies we tested sandblasted surface implants (SLA) showing a regular and softly shaped profile with moderately deep cavities very similar to the osteocyte lacunae which were found positively influencing growth and metabolic activity of osteoblasts *in vitro*. Implant design has been modified over time in order to achieve a better spreading of stress and a higher rate of osseointegration. Nowadays endosseous screw-shaped titanium dental implants dominate the dental implant market. In these implants the mechanical friction of the threads with the host bone cavity is able to ensure the primary stability as histomorphometrically observed in our recent research on threaded implants in sheep cortical bone. However, clinicians have reported that success rates for implants is markedly reduced when implants are placed in poor quality bone. This preliminary *in vivo* study on a dog lower jaw aimed to investigate the hypothesis that implant stability in low density bone may be influenced by a combination of macroscopic and microscopic features.

We compared two implant types exhibiting similar microroughness surface but different thread profile with regard to their early quantitative relation to host bone. Experimental implants (Fig.1a,b) were conic, screw-shaped, rounded-apical-end, titanium implants manufactured by Or-Vit (Castelmaggiore-Bologna, ITALY). All implants measured 4 mm in maximum outer diameter and 10 mm in length. The screws were provided of three longitudinal grooves The surface was modified by an sandblasting procedure with alumina oxide particles (Al_2O_3SLA). The implants were designed with two different profiles: (1) "passo fine" implant (PF), with a 0.5mm distance between the crests of two consecuitve threads and (2) "passo largo" implant (PL), with a 1.5mm distance between the crests of two consecuitve threads. The *in vivo* study was performed on one female Belgian Shepherd Dog. Second, third and fourth mandibular premolars were carefully extracted bilaterally. After a eight weeks healing period two implants of each screw design type were placed in a alternate order in the right and left side of the jawbone (Fig.1c). Five hours after surgery the animal was sacrificed (Day 0). Radiographic, histological, morphometrical and ultrastructural analysis were performed.

This preliminary study has to be considered as the starting point of an in-progress research by which we aim to
investigate the influence of implant geometry on osseointegration process in low density bone. The final goal is to correlate morphological results with the functional data expressing the clinical successful retention of implants at different bone quality sites.

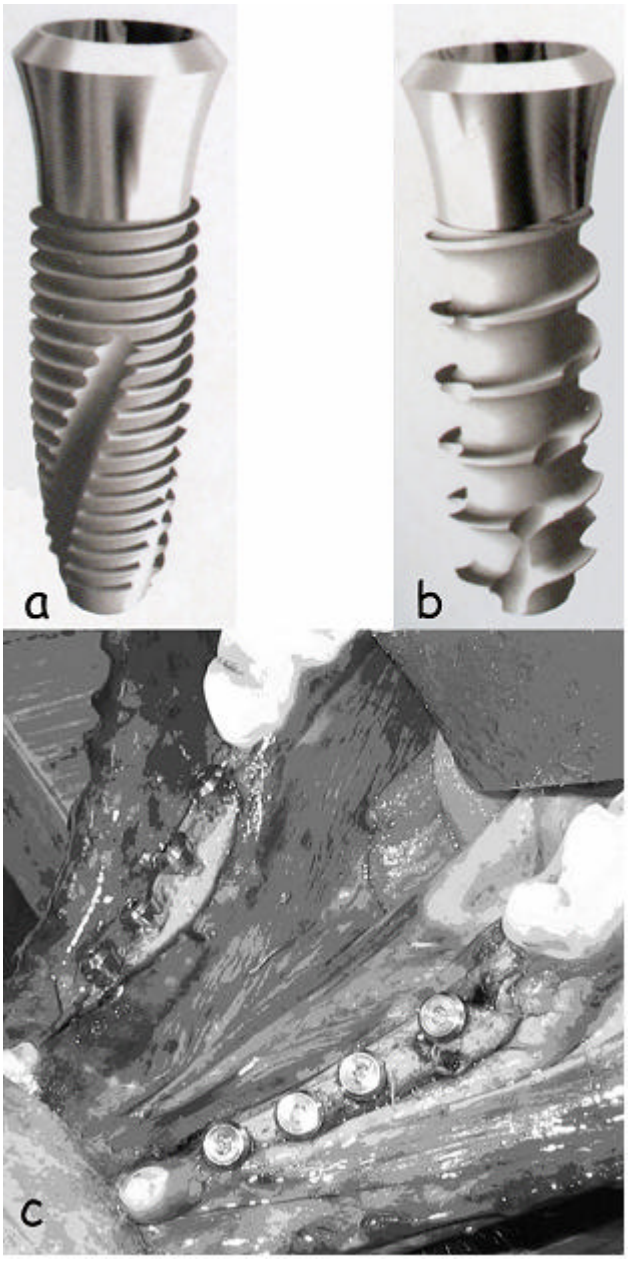

Fig.1 - Experimental implants: "Passo fine" (a) and "Passo largo" (b) implants. Surgical implantation of "Passo fine" and "Passo largo" in an alternate order in the jaw bone after 8 weeks healing period (c)

IMPROVED CELL GROWTH BY BIO-OSS/PLA SCAFFOLDS AS A BONE SUBSTITUTE

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INTRODUCTION. Various natural and synthetic bioresorbable materials or a combination of them, are being investigated for design and construction of scaffolds for the engineering and regeneration of a wide variety of tissues.[1] Bio-Oss is a natural bone substitute made of the mineralized portion of bovine bone; it is used mainly to fill bone defects in periodontal and maxillofacial surgery and permit reossification. Bio-Oss through its trabecular architecture, pore size (300-500 μm), and high porosity (70-75%) promotes the invasion of blood vessels and bone cells, thereby inducing revitalization and ossification of the defect. Nevertheless, the cell proliferation rate does not result as well if compared to other biomaterials in use[2]. Polyesters such as poly (lactic acid) PLA are the most popular synthetic polymers considered for scaffold applications. In particular regarding bone tissue engineering, the development of materials comprising a biodegradable polymeric phase and a bioactive inorganic phase, is seen as a promising approach for scaffold production. The objective of this study was to investigate the surface modification of a natural bone substitute, Bio-Oss, coated with a synthetic polymer poly-D,L-lactide (PLA), in order to improve cell growth[3].

MATERIALS and METHODS Bio-Oss scaffolds were provided by the manufacturer Geistlich-Pharma-(Wohlusen, Switzerland) and are commercially available. PLA coated scaffolds were prepared by a casting method with an interpenetrating film of poly D,L-lactide (PLA4M). *Cell culture*: the human osteosarcoma cell line SAOS-2 was obtained from the American Type Culture Collection (HTB85, ATCC). The cells were cultured in McCoy's 5A modified medium with L-glutamine and HEPES supplemented with 15% fetal bovine serum, 2% sodium pyruvate, 1% antibiotics, 10^{-8}M dexamethasone, and 10mM β-glycerophosphate (Sigma-Aldrich). The cells were cultured at 37°C with 5% CO_2, routinely trypsinized after confluence, counted, and seeded onto the scaffolds. *Cell seeding*: a cell suspension of $1x10^7$ in 200 μl was added onto the top of each scaffold. The cells were incubated at 37°C in 5% CO_2 for 15 days. A set of polyclonal antibodies was selected for ELISA assays. Rabbit polyclonal antisera (anti-type I and III collagen, anti-decorin, anti-osteopontin, anti-osteonectin, and anti-osteocalcin were kindly provided by L.W. Fisher (National Institutes of Health, Bethesda, MD). At the end of the culture period, the extracellular matrix (ECM) extraction was carried out in order to evaluate the amount of the ECM constituents over the scaffold surface. A confocal laser scanning microscopy (CSLM) study of the Bio-Oss/PLA scaffolds (CSLM) was performed to evaluate immunolocalization of bone proteins. Bio-Oss/PLA4M scaffolds, before and after incubation with SAOS-2 cells, were observed by scanning electron microscopy (SEM).

RESULTS A Gel Permeation Chromatography analysis performed after 15 days of incubation showed that the average molecular weight (Mw) decreased by 19.39 % for PLA4M raw material and by 14.31% for PLA4M coating. This behaviour is confirmed by the corresponding decrease in molecular number (Mn) and in polydispersity index (Pi). The percentage of the Mw decrease upon incubation is consistent with the polymer degradation behaviour as reported in the literature. In order to evaluate the amount of the ECM constituents over the scaffold surface, an ELISA assay of the extracted matrix was performed at the end of the culture period. As expected, most of the typical bone proteins were detected, with type-1 collagen being the most representative. The CSLM study of the Bio-Oss/PLAscaffolds showed the immunolocalization of type-I collagen , osteopontin and osteocalcin. All together these data were in agreement with results of SEM analysis, where after 15 days of incubation SAOS-2 attached and spread well over the surface of porous Bio-Oss/PLA4M scaffolds, as shown in figure 1. SAOS-2 cellular process cover almost the entire material surface indicating the biocompatibility of the polymer.

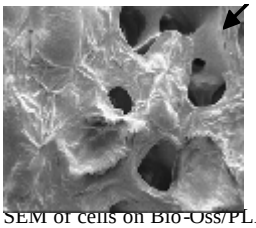

Fig.1 SEM of cells on Bio-Oss/PLA4M

CONCLUSIONS. In this study SAOS-2 cells adhere to biocompatible polymer layers (4MPLA) on the Bio-Oss surface. Porous three-dimensional tissue engineering scaffolds act as temporary ECM for the physical support of cells and to allow manipulation of various cell functions, such as cell adhesion, cell growth, and cell differentiation. This study was conducted to develop and characterize *in vitro* a novel scaffold consisting of Bio-Oss and PLA; analysis of the construct morphology, degradation behaviour and characterization of the produced extracellular matrix, osteocalcin, osteopontin and collagen I, III measurement confirm the good bioactive properties of the scaffolds.

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PHYSICALLY AUGMENTED COATING OF MACRO-ROUGH TITANIUM SURFACE WITH HUMAN SAOS-2 OSTEOBLASTS AND BONE MATRIX

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INTRODUCTION. The early osteointegration of a macro-rough or a micro-rough metallic surface depends mainly on the ingrowth of the bone tissue onto the material surface.
Ti-alloy implants with a rough surface are widely used in orthopaedics due to their good mechanical properties and biocompatibility. Titanium surfaces characterized by macro-roughness have been studied with the focus on enhancing the bone apposition onto the implant.
Aiming at an accelerated and enhanced *in vivo* osteointegration of a macro-rough titanium surface during the early postimplantation period, we show a particular "biomimetic strategy" that consists in the surface modification of the biomaterial with proliferated bone cells and their extracellular matrix produced *in loco*.
In addition, during the culture period we have applied an electromagnetic wave because the bone cell function can be physically modulated in terms of mitosis and differentiation (1).

MATERIALS AND METHODS. Disks (diameter, 12 mm; height, 4 mm) were sintered from the titanium alloy Ti6Al4V powder. The resulting net had square holes with side of 800 ? m (Lima) (Fig. 1).
Human osteosarcoma cell line SAOS-2 was cultured in McCoy's 5A medium, supplemented with 15% fetal bovine serum, 2% sodium pyruvate, 1% antibiotics, 10^{-8} M dexamethasone, and 10 mM ? -glycerophosphate. The cells were cultured at 37°C with 5% CO_2. A cell suspension of 4×10^5 cells in 100 ?l was added onto the top of each disk. An electromagnetic (frequency, 75 Hz; magnetic field, 2 mT; duration, 24 h per day) stimulus was applied to the seeded disks for 22 days.

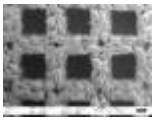

Fig. 1 - Net of sintered titanium, 10X, bar=100 ? m.

RESULTS. The SEM images revealed that, due to the physical stimulation, the cells proliferated over the available titanium surface (data not shown).
These observations were confirmed by the measure of the DNA content after 22 days: in the control culture the cell number per disk grew to $8.5\times10^6 \pm 4.7\times10^4$ and in the electromagnetic culture to $17.5\times10^6 \pm 9.3\times10^4$ with $p<0.05$.
The immunolocalization of type-I collagen showed a more intense fluorescence in the physically cultured disks, revealing the stimulation effects in terms of higher cell proliferation and more intense building of the extracellular matrix (Fig. 2).
To evaluate the amount of bone matrix over the disk surface, an ELISA of the extracted matrix was performed: at the end of the culture period, in comparison with the control culture, the physical stimulus greatly increased the coating with type-I collagen ($p<0.05$) (Tab. 1).

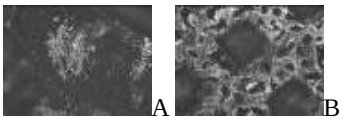

Fig. 2 - Immunolocalization of type-I collagen (green) in the control (A) and electromagnetic (B) cultures, 25X, bar=400 ? m.

Tab. 1 - Type-I collagen coating in fg/(cell×disk)

Control	Electromagnetic
645.23 ± 35.76	9852.24 ± 262.81

DISCUSSION. The aim of this study was the surface modification of a titanium net with extracellular matrix and osteoblasts to make the biomaterial more biocompatible for the bone repair *in vivo*.
To enhance the coating of the biomaterial, an electromagnetic wave was applied to the seeded disks. The physical stimulus increased the cell proliferation around 2-fold. The physical stimulus also increased significantly the extracellular matrix synthesis: in comparison with the control culture, the coating with type-I collagen was enhanced around 15-fold.
The use of a cell line showed the potential of the physical stimulus; nevertheless, a better result could be obtained with autologous bone marrow stromal cells instead of SAOS-2 osteoblasts for total immunocompatibility with the patient.
In conclusion, we could theorize that the cultured biomaterial could be used fresh, that is, rich in autologous cells and matrix, or after sterilization with ethylene oxide, that is, rich only in autologous matrix in order to handle a simpler storable tissue-engineering product for bone repair.

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OSTEOGENIC DIFFERENTIATION OF ADIPOSE TISSUE-DERIVED STEM CELLS CULTURED ON TRABECULAR TITANIUM SCAFFOLDS

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INTRODUCTION. The use of stem cells in regenerative medicine is an appealing area of research that has received a great deal of interest in recent years. Different kinds of stem cells have been used for 3D cultures in tissue engineering applications.
The population called Adipose Tissue-Derived Stem Cell (ADSCs) share many of the characteristic of its counterpart of marrow including extensive proliferative potential and the ability to undergo multilineage differentiation along classical mesenchimal lineages: adipogenesis, chondrogenesis, osteogenesis, and myogenesis. They are of great interest due to their accessibility and expandibility.[1] The acquisition of adipose tissue is much less expensive than bone marrow, with less invasive withdrawal; moreover , it is available in greater quantities. A clinically relevant number of stem cells can be extracted from isolated adipose tissue since it has higher stem cell proliferation rate than BMSCs[2]. Under osteogenic conditions, ADSCs are observed to express genes and proteins associated with the osteoblast phenotype, including alkaline phosphatase, type I collagen, osteopontin, osteonectin, osteocalcin, bone sialoproteins, RunX-1, BMP-2, BMP-4, BMP receptors I and II, PTH receptor. ADSCs are also able to form mineralized matrix in vitro in both 2-D or 3-D long term osteogenic cultures [3].
Aim of this investigation is the evaluation with biochemical and morphological methods of the adhesion and differentiation of ADSCs grown on Trabecular Titanium scaffold.

MATERIALS AND METHODS. Trabecular Titanium scaffolds (Ti6Al4V) were provided by manufacturer LIMA-LTO Medical System (San Daniele del Friuli, Udine, Italy). Previous studies have shown that the minimum pore size to improve osteointegration is 300 µm. The average diameter of the cell pores used in Ti6Al4V construct is 640 µm and the structure has an average porosity of 65%.
ADSCs were obtained from subcutaneous adipose tissue of healthy donors during surgical procedures, after informed consent obtained from the patients. Cells were suspended in
expansion medium (DMEM F12-HAM plus 10%FBS, antibiotics and amphotericiyn), seeded in flasks and cultured at 37°in 5% CO_2 humidified atmosphere until subconfluence. Adherent cells were then trypsinized and $1x10^6$ cells were inoculated on trabecular titanium scaffold and incubated at 37°in 5% CO_2 humidified atmosphere with osteogenic medium (DMEM F12-HAM plus 15% FBS, 10mM ßglycerophosphate, 100nM dexamethasone, 50µM acid ascorbic, antibiotics and amphotericyn). *Osteogenic differentiation* was evaluated after 21 days with the following methods: Von Kossa staining for qualitative assessment of mineralized matrix formation; alkaline phosphatase activity; expression of osteopontin, osteocalcin, and type I collagen with RT-PCR. SEM was performed on titanium scaffolds after 15 and 21 days of culture. X-ray microanalysis of the samples was run to detect the presence of elements and their location within the scaffolds after 15 and 21 days of cell culture.

RESULTS. After 21 days of culture in osteogenic medium Von Kossa staining was positive and alkaline phosphatase activity showed an 80% increase. Moreover, ADSCs expressed osteopontin, osteocalcin and type-I collagen that are specific proteins of osteoblastic phenotype. SEM observations showed cells attached over the surface of porous scaffold (Fig. 1) and embedded in a plentiful extracellular matrix (ECM) with homogeneous appearance and well organized. Interaction between ECM proteins and cells can directly control cell behaviour such as attachment, migration, proliferation, differentiation and apoptosis. X-ray microanalysis showed the presence of calcium and phosphorus, inferring that calcium phosphate had formed.

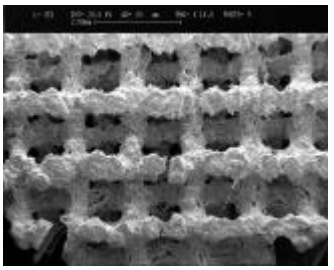

Fig. 1 SEM of ADSCs grown on trabecular titanium scaffold after 15 days of culture in osteogenic medium

CONCLUSIONS. We observed that ADSCs can adhere and proliferate on trabecular titanium scaffold surface and inside the pores, and differentiate towards osteoblastic-like cells that are embedded in a homogeneous extracellular matrix. The three dimensional scaffold culture systems may provide osteoblast–like cells with an environment more similar to *in vivo* conditions than the monolayer culture does[4]. Therefore ADSCs showed to be an ideal source of cells for tissue engineering.

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TUMOR MICROENVIRONMENT AS TARGET IN CANCER PREVENTION; MATRIX METALLO-PROTEASES AND BEYOND

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Tumor progression depends on the formation of new vessels (neo-angiogenesis) and cellular invasion and metastasis. Besides cancer cells, numerous factors and cells participate to these processes (1).
Matrix metallo-protease 2 and 9, promoting extracellular matrix degradation, favourite pathological process such as inflammation, angiogenesis and tumor invasion (2); these enzymes are frequently overexpressed in cancer and inflammation (2).

Recent data from studies on the tumor microenvironment suggest that it is an integral and essential part of the cancer (1). The tumour microenvironment is a complex of many cell types, including endothelial cells and precursors, pericytes, smooth-muscle cells, fibroblasts of various phenotype, myofibroblasts, neutrophils, eosinophils, basophils, mast cells, T, B and natural killer lymphocytes, and antigen-presenting cells such as macrophages and dendritic cells. All these cells have a crucial role in tumor progression, stimulating the formation of the blood vessels (3).

We sought to identify molecules, such as flavonoids, antioxidants and retinoids, and pathways to prevent tumor development by targeting cancer invasiveness (4), the microenvironment (3) and inflammatory angiogenesis (1).
We have demonstrated that epigallocatechin-3-gallate (EGCG), the most abundant polyphenol found in dried tea leaves, has anti-invasive properties as a potent inhibitor of the matrix metallo-protease 2 and 9 without impairing cytoskeleton and motility (4). EGCG also inhibits the activity of Proteinase-3, a serine-proteinase mainly expressed by polymorphonuclear leukocytes, which can degrade a variety of extracellular matrix proteins and activate matrix metallo-protease 2 and 9 (5).

The chemopreventive flavonoid curcumin derived from curry has an anti-metastatic effect on breast cancer cells line MDA-MB-231, inhibiting the pro-survival NF-kB pathway and decreasing the expression of the major metallo-proteases (6).
Furthermore, we have shown that Hyperforin, a polyphenol-derivates of St. John's wort (Hypericum perforatum), blocks leukocyte elastase-triggered activation of matrix metallo-protease 9 in polymorphonuclear cells (7).

Many of the studied molecules contrast inflammation-associated angiogenesis by targeting cells in the tumor microenvironment, inhibiting the recruitment and/or activation of endothelial cells and innate immune cells and suppressing the IkB/NF-kB signalling pathway (1) .
Natural compounds have important properties useful in cancer prevention and therapy; deeper investigations are necessary to improve the knowledge in this field.

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CONTRIBUTION OF CONNECTIVE AND EPITHELIAL TISSUE COMPONENTS TO THE MORPHOLOGICAL ORGANIZATION OF CANINE TRICHOBLASTOMAS.

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The present paper aimed at investigating the architectural organization of canine trichoblastoma in relationships with distribution patterns of epithelial /connective tissue components and the expression of ß-Catenin, a structural between cells, anchoring the actin cytoskeleton, regulating normal cell growth and behaviour, and regulating epithelial architecture and polarity of cells and tissues. Trichoblastomas consti-tute a class of < benign tumour derived from or reduplicating the primitive hair germ of embryonic follicular development > (1) and represent about 25% of all canine epithelial skin neoplasms (2). They are classified into ribbon (RT), trabecular,/granular cell (TT/GT) and spindle cell (ST) types. However, trichoblastomas are not exclusively epithelial tumours but heterogeneous biological systems arising from epithelial/mesenchymal interactions (3). In these neoplasms the epithelial component appears to be equivalent to the hair germ and the mesenchymal component to represent the dermal papilla, both essential for hair follicle development (3). While the interactions between stroma, extracellular matrix and the epithelium compartment have been extensively examined in other mammalian tissues (4,5,6,7), the complex mesenchymal /epithelial interactions and the link between morphogenetic and molecular cell signals that may be taking place in trichoblastomas remain poorly understood.

Methods. Conventional histological sections were prepared from canine trichoblastoma tissues. Immuno histochemistry: deparaffinized sections were incubated with monoclonal antibody anti ß-catenin and stained with the peroxidase/antiperoxidase procedure. Morpho metric analysis: it was carried out by using Image J in order to determine the proportion of epithelial and mesenchymal components in tumour tissues. Ten fields at 10x magnification were randomly selected from histological sections of each tumour, (a single examined field had a surface area of 0.28 mm^2) and superimposed with a square lattice grid of one hundred points (Pt). The number of points overlapping each tissue component was counted (32) and the relative tissue component distribution (%) obtained by the following equations: $[V_v]_e = 100 \times P_e / Pt$; $[V_v]_m = 100 \times P_m / Pt$ were $[V_v]$ indicates the volume density expressed as the ratio of points (P_e ; P_m) overlapping on the epithelial (e) and mesenchymal (m) tissue components respectively to the total number of lattice points (Pt). Fractal analysis: masks and outlines of the epithelial tumour components were segmented from grey level threshold pictures randomly taken (at10x) from trichoblastoma histological sections. The fractal parameters were determined from the above mentioned digital pictures using the FANAL++ program (8). The slope of the straight line, drawn within the scaling interval analytically estimated, yields the fractal dimension [FD] characteristic of each profile.

Results. Morphometric results indicated that the relative amount of the mesenchymal stroma expressed as [Vv] M was higher ($p<0.001$) in the RT type (46% ± 2%) compared to ST and TT/GT types accounting for 31% ± 5% and 33% ± 3% respectively. All the examined tumours showed epithelial components with irregular and self-similar properties documented by characteristic fractal dimension values [FD] estimated either from tumour masks and outlines. Tumour masks analysis unexpectedly revealed that RT trichoblastomas were less irregular with lower FD values (1.75 ± 0.01), significantly different ($p<0.001$) from FDs of the ST and TT/GC types accounting for 1.78 ± 0.03 and 1.85 ± 0.02 respectively. Trichoblastoma outlines showed absolute FD values lower than those obtained from tumour masks and revealed statistically inadequate to enable any histological discrimination. ß-catenin occurred in the cytoplasm and in the nucleus of both mesenchymal and epithelial neoplastic cells, while its expression pattern (nucleus, cytoplasms, or both) was closely related with the growth pattern morphology and the architectural irregularity of trichoblastoma types, as documented by the FD which progressively increased from RT, ST up to TT/GT.

Conclusions. Canine trichoblastoma could represent a reliable model to unravel the morphogenetic dynamics underlying the neoplastic transformation and could provide in quantitative terms useful informations about the link between molecular, cellular and tissue changes during the tumour development.

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S-100 CALCIUM BINDING PROTEINS AS POTENTIAL MARKERS FOR BREAST CANCER METASTASIS

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The S-100 family of calcium-binding proteins includes about 20 members of low molecular weight characterized by two consecutive EF hands domains. They make interactions with cellular target proteins in a calcium-dependent manner; therefore they are thought to regulate a variety of physiological functions, such as cell proliferation, signal transduction, cell adhesion, motility as well as cancer metastasis. There is increasing evidence that altered expression of S100 family members occurs in many cancers including breast, lung, bladder, kidney, thyroid, gastric, prostate and oral cancers. Many of S100 proteins appear up-regulated in tumours and associated with progression, probably through interactions with matrix metalloproteinases or by acting as chemoattractants. Some of them, as S10A2, S10A1 and S10A9, have ambiguous roles, being documented as tumour suppressors in some cancers and promoters in others [1]. This demonstrates the need of further information to discover precise roles of these proteins in cancer. The aim of present research was to investigate the occurrence of S100 family members in breast cancer tissues at proteomic level.

To this purpose, we examined 100 ductal infiltrating breast cancer specimens, obtained from surgical resection, with patients consensus. Aliquots of breast cancer tissue and its adjacent non-tumoral tissues were immediately frozen in liquid nitrogen and stored at -80 °C until use. The patients did not receive any cytotoxic/endocrine treatment prior to surgery. In each cases, non-tumoral tissue was located at least 5 cm away from the primary tumor. Diagnosis was confirmed histopathologically.

Sample preparations and 2D-IPG were performed as described [2]. Protein identification was accomplished by protein sequencing, either N-terminal or internal by mass-spectrometry (Maldi-Tof) [3].

We have recently reported the comparative proteomic profiles of a set of paired tumoral and non-tumoral tissue samples obtained from patients with ductal infiltrating breast cancer [4]. As expected, the contribution of cellular proteins to the proteomic complexity, was much higher in the tumor extracts than in their paired non-tumoral counterparts. Thereafter, the study was extended to a larger number of cases. In order to perform comparative proteomics, data were normalized in each map for actin content, as index of cellularity of the sample [5].

In figure 1 is reported the image of a gel window including the protein spots corresponding to the detected S100 protein family members. These are: S10A4 (metastasin), S10A6 (Calcyclin, Prolactin receptor-associated protein), S10A7 (psoriasin), S10AB (S100A11), S10AD (S100A13).

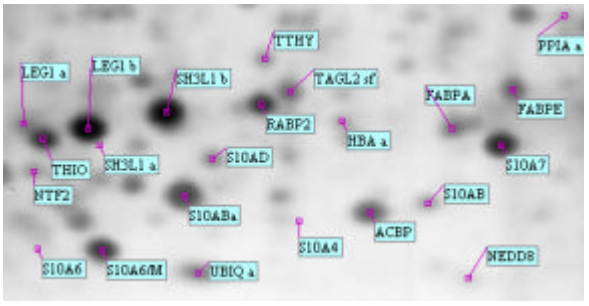

Figure 1.Gel window (p*I* 4.90-6.45; kD 15.32- 8.33) of representative proteomic map of a breast cancer tissue showing the collection of S100 proteins.

Among the S100 occurring in our pathological samples, the S10A7 was one of the more sporadic, being expressed only in about 20% of the patients so far studied. However, when present the protein was very often of remarkable intensity. Immunological validation, by western blot analyses on tissue extracts, confirmed the obtained results.

Among the predicted functional partners of S10A7 (http://string.embl.de/) are other S100 proteins (S10A1 and S10A10), the fatty acid-binding protein 5, interleukin 22 and its receptor, and two oncogenes, HRAS and Jun with one of its co-activators. This interactom for S10A7 protein appears of great interest and encourage the investigation for wide-scale clinical correlations to define the possible role of the S10A7 protein and its partners in breast cancer metastasis.

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ANTI-ONCOGENIC ROLE OF DECORIN: IDENTIFICATION OF NEW MARKERS.

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Decorin is a prototype member of the small leucine-rich proteoglycan (SLRP) family widely distributed in the extracellular matrices of many connective tissues.
In the last decade, a number of investigations pointed out the potential antioncogenic role of decorin, as growth-inhibitor of different neoplastic cells and putative anti-metastatic agent [1,2].
Our previous collaborative studies have reported the effects of ectopic decorin on proteomic profile and phenotypic responses of breast cancer cell line 8701-BC [3]. In order to investigate further the effects of decorin on the gene expression of the 8701-BC cells, we performed combined analyses at genomic and proteomic level.
For proteomic analyses, parental cells and transfected clones were lysed in RIPA buffer to obtain proteic extracts. Extracts were solubilized in ISOT buffer, protein concentration was determined by the Bradford method and an aliquot of 1.5 mgr was subjected to IPG-2D electrophoresis. Proteomic maps were stained with Coomassie Blue. Selected spots were subjected to tripsin digestion and derived peptides were analyzed by a Maldi-Tof mass spectrometer.

For genomic analyses, cells were collected at proper time of culture, and subjected to lysis for the extraction of total RNA, using the Trizol reagent (Invitrogen). A panel of oligo GEarrays (Super Array Inc.) was used to evaluate differential gene expression between parental 8701-BC cells and their decorin-transfected clones. These arrays included 264 genes selected among breast cancer clinical biomarkers.
New proteins detected in this study include metabolic enzymes, signal transductors, pro-apoptotic factors, cell cycle regulators and cell-matrix interactors.

Genomic approach provided new information on differential gene expression induced by decorin transfection. In spite of the limited possibility for directly comparing individual genes and corresponding proteins in a genomic/proteomic approach, present investigation gave convergent results regarding the functional categories of genes and proteins modulated by decorin..

Results of present study show increase of expression of proteins and gene transcripts which play a pivotal role in cell cycle regulation and in pro-apoptotic stimulation. Conversely, evident decrease of some glycolytic enzyme, anti-apoptotic factors and cell-matrix interactors was observed..
Collectively, data so far obtained from genomic and proteomic analyses, converge in the evidence that decorin effects on neoplastic cells are strongly directed towards the reversion to less aggressive neoplastic phenotype.

These effects are also testified by the impressive morphological changes in cell shape, morphology and cell-cell interactions. As shown in figure 1A 8701-BC cells display highly irregular morphology and tend to form multilayered colonies, typical of malignant cells. Decorin treatment induces a reversion towards a more regular and polaryzed cell growth (figure 1B). These data confirm and strengthen the postulated role of decorin as anti-oncogenic factor.

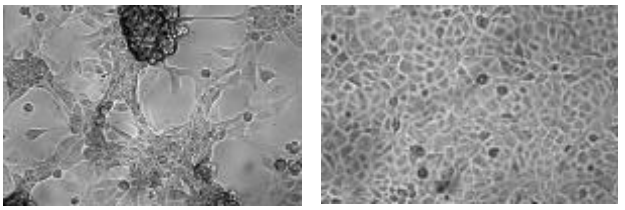

Figure 0. Phase-contrast micrograph of 8701-BC parental cells (A) and decorin-transfected clone (B) showing the different modality of cell growth and morphology.

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STRUCTURE OF KNEE MEDIAL COLLATERAL AND PATELLAR LIGAMENT IN RAT

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In tendons and ligaments extracellular matrix collagen is the major component transferring forces within these tissues. Generally collagen fibrils run straight and parallel to the main axis, packed to form collagen fibres, with periodic crimps. Collagen crimping imparts important biomechanical properties on the connective tissue in which it is found (1).

Although tendons and ligaments have similar composition, they show different structure in relation to their different functional role (2). Similarly ligaments subjected to different mechanical requests presumably show different structure.

Aim of this study is to investigate ultrastructure and arrangement of collagen fibrils in two ligaments submitted to different forces: knee medial collateral ligament (MCL) and patellar ligament (PL). The ligaments were isolated from 10 female Sprague–Dawley to be processed for polarized light microscopy (PLM) and scanning electron microscopy (SEM). Sagittal sections of the tissues were analyzed for morphometric analysis to measure the number of crimps, the width of crimp top angle and the crimp base length.

In MCL, PLM and SEM analyses revealed two distinct regions displaying a different array of collagen fibre bundles. The core of the ligament consisted of collagen fibres running straight and showing the typical periodic crimps. Some of them appeared in a planar array whereas others showed a rotation of few degrees on their main own axis. The peripheral areas showed collagen fibre bundles arranged in an oblique patterrn (Fig. 1a).

In PL collagen fibre bundles appeared more uniformly arranged than in MCL: they mostly ran parallel to its own long axis showing planar crimps with no differences between core and periphery (Fig. 1b).

The crimp number in MCL (9.7±1.9) appeared slightly higher as compared to PL (6.1±2.8), whereas the crimp base length was almost two times higher as wide in PL (73.2±27.5) than in MCL (37.2±9.0). The mean crimp top angle in MCL (142.9±8.7) was almost the same as in PL (146.2±12.2).

The central longitudinally arranged collagen fibre bundles in MCl, similarly to all collagen fibrils in PL, are presumably submitted to uniaxial tensile loading. Differently the multi oriented collagen fibres bundles in the peripheral region of MCL are arranged to resist multidirectional loadings. These observations are in agreement with previous transmission electron studies demonstrating in the core part of MCL large fibrils presumably exhibiting a better strength (3). In the peripheral MCL region the collagen fibrils with a small diameter were obliquely oriented. The straight packed collagen fibre arrangement in PL, similary to the collagen array in all tendons, suggest that PL is submitted to uniaxial forces.

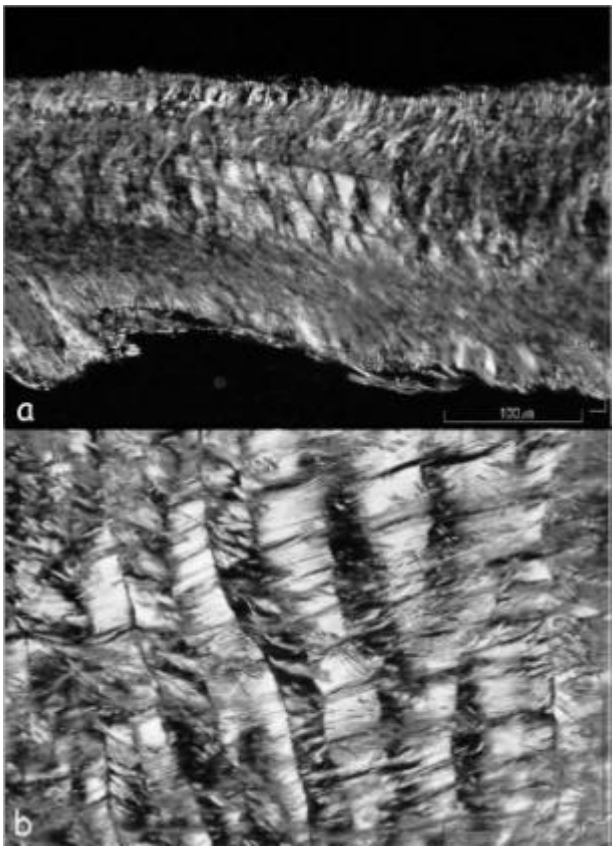

Fig. 1- PLM: a) MCL and b) PL

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CHONDROITIN SULPHATE, HEPARAN SULPHATE AND HYALURONAN REDUCE CYTOKINE PRODUCTION BY TOLL-LIKE RECEPTOR-4 MODULATION IN MOUSE CHONDROCYTES STIMULATED WITH LPS

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Recent findings suggest that glycosaminoglycans (GAGs) such as hyaluronan (HA), chondroitin-4-sulphate (C4S), chondroitin-6-sulphate (C6S) and heparan sulphate (HS) may protect cells from oxidative stress (1). It was also reported that HA and C4S were able to reduce cell damage by inhibiting the nuclear factor kappaB (NF-kB) and caspases activation that in turn determines cell apoptosis (2-3). Toll-like receptors (TLRs) are phylogenetically conserved receptors involved in the innate immune response. Because ligand recognition by TLRs elicits strong activation of pro-inflammatory cytokines, the role of TLRs in the exacerbation of the inflammatory response has been postulated (4). Lipopolysaccharide (LPS), the principal component of the outer membrane Gram-negative bacteria, is able to active TLR-4 complex inducing specific signalling pathways, such as the myeloid differentiation primary response protein (MyD88) and the tumor necrosis factor receptor-associated factor 6 (TRAF6). These pathways involve the phosphorylation of several kinases that in turn led to liberation of NF-kB/Rel family members into the nucleus. NF-kB then binds to the promoter region of immune and inflammatory genes including iNOS for transcriptional regulation. Since previous data showed that HA and HS were able to interact with TLR-4 (5-6), the aim of this study was to investigate whether the anti-inflammatory activity exerted by GAGs may be due to an antagonist effect on TLR-4 receptor in a model of LPS-induced increase of pro-inflammatory cytokines in mouse articular chondrocyte cultures.

Chondrocyte stimulation with LPS (10 ?g/ml) for 24 hours increased TLR-4, MyD88 and TRAF6 mRNA expression and their related proteins, induced NF-kB activation and finally generated high levels of tumor necrosis factor alpha (TNF-?), interleukin beta (IL-1? ? and the inducible nitric oxide synthase (iNOS), evaluated in terms of gene expression and protein production.

The treatment of chondrocytes with two different doses (0.5 mg/ml and 1.0 mg/ml) of HA, C4S, C6S and HS produced various effects: HA reduced MyD88 and TRAF6 levels and NF-kB activation with the higher dose only, and exerted a very low anti-inflammatory effect; C4S and C6S significantly inhibited MyD88, TRAF6 production and NF-kB activation and the inflammation mediators TNF-? ?nd IL-1? ?although C6S exerted less evident effect than C4S; iNOS expression and activity were also significantly reduced; HS, like C4S, was able to significantly reduce MyD88, TRAF6 and NF-kB activation and inflammation.

These results indicate that the inhibitory effect exerted by GAGs on NF-kB activation may follow the block of TLR-4, although a direct action on NF-kB can not be excluded.

Since GAGs are able to bind a variety of biological molecules, especially proteins, the block of TLR-4, together with their antioxidant activity and an eventual direct inhibition of NF-kB, may represent a further step of GAG fine modulation of the inflammatory mechanism.

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Added GAG (+LPS)	TLR-4	MyD88	TRAF6	TNF-a	IL-1b	MMP-13	iNOS
HA, 0.5	3.3	2.2	5.6	5.4	6.7	8.5	6.7
HA, 1.0	20.0	16.0	15.6	17.3	13.3	17.0	13.4
C4S, 0.5	25.0	18.1	16.8	20.2	18.4	25.5	19.2
C4S, 1.0	29.0	17.6	23.2	24.7	23.6	34.1	25.8
C6S, 0.5	16.6	16.0	11.2	14.9	14.7	20.8	20.8
C6S, 1.0	22.8	18.1	19.2	19.7	19.9	26.5	25.8
HS, 0.5	22.0	16.0	16.8	23.2	17.7	23.6	15.0
HS, 1.0	27.2	22.4	24.8	27.7	25.8	30.2	24.2

Table 1 - The effect of glycosaminoglycan administration, at the doses of 0.5 mg/ml and 1.0 mg/ml, on LPS activation of TLR-4 in mouse chondrocytes. % reduction of the increase with LPS treatment obtained by contemporary GAG addition in terms of RNA expression.

ENDOGENOUS FIBRONECTIN MODULATES COLLAGENDEPENDENT SPREADING AND PROPLATELET FORMATION BYHUMAN MEGAKARYOCYTES

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The signals that initiate and regulate proplatelet formation (PPF) are still poorly understood (1), but the characteristics of the microenvironment surrounding megakaryocytes may play an important role in supporting platelet production (2). In this work we have analyzed the role of different adhesive proteins in megakaryocyte (MK) spreading and proplatelet formation by human cord blood derived haemopoietic progenitor cells.

MKs were differentiated from cord blood derived CD34+ cells for 12 days. Mature MKs were plated onto glass coverslips coated with different adhesive proteins, MK spreading and PPF were evaluated by phase contrast and fluorescence microscopy upon cell staining with phalloidin, anti-tubulin and CD41 antibodies.

We found that adhesion of megakaryocytes to fibrillar type I collagen inhibited PPF, but not MK spreading that was maintained in 16 hour incubation and modulated by translocation of endogenous fibronectin to cell periphery. This process was strictly dependent on the adhesive substrate: in fact immunofluorescence staining of spreaded MKs on collagen I, but not on fibrinogen, showed an exposure of endogenous fibronectin to the cell membrane. The interaction of MK with type I collagen seemed to be influenced by its fibrillar structure and biochemical properties. Therefore we showed that chemical modification of collagen I permitted PPF, while completely inhibited MK spreading in 16 hours incubation.

In conclusion in this work we report that endogenous fibronectin finely regulates PPF and MK spreading on type I collagen .This new mechanism highlights how the interaction with collagen influences megakaryocyte behaviour and platelet release.

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EVALUATION OF CELLULAR RESPONSE OF BREAST CANCER CELLS GROWN ON DISTINCTIVE COLLAGEN SUBSTRATES.

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Breast cancer is one of the most frequent carcinoma for women and at same time one of the less predictable, mainly because it includes various forms that behave differently among patients.
A crucial difference between benign and malignant breast tissues is that the former are limited by a basement membrane for undefined periods of time and do not infiltrate or form metastases, whereas the latter acquire the ability to invade the underlying basal lamina and its adjacent stroma.
Several lines of evidence suggest that perturbation of stromal-epithelial interactions are directly or indirectly responsible for the onset of cancer progression.
During the years, our research group has produced several evidence concerning stromal changes in cases of infiltrating carcinomas, as well as biological effects of different collagen types, which likely enter in contact with neoplastic cells during the invasive growth [i.e. 1, 2,].
In the present study we further investigated by a proteomic approach the effect type V and type I trimer (OF/LB) collagens. We utilized an in vitro model of cells isolated from a primary DIC, the 8701-BC line [3]. As control condition, we cultured cells in the presence of type IV collagen, that represents a physiological condition of growth for epithelial cells. Reconstituted collagen substrata, were prepared according to our previously published protocols [4,5].

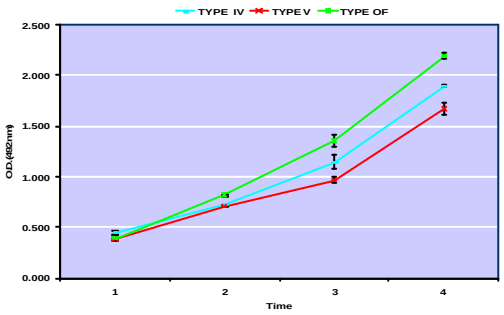

Fig1: Proliferation assay of 8701 BC cells seeding on IV, V, LB/OF collagen substrates.

When monitoring the proliferation rate of the cells cultured on IV, V and OF/LB substrates during a culture time of 7 days, a restrictive effect was observed for type V collagen, in opposition to a promoting effect exerted by the oncofetal collagen. The former induced 13% decrease, while the latter 14% average increase of cell number at 7 days/versus IV substrate.
In order to evaluate proteomic effects exerted by different substrates, cells grown until confluence (7 days from seeding) were harvested and processed for the proteomic assay as previously described [6].

For the comparative analysis of protein expression levels in the different cell-growth conditions, quantification of proteins was performed by measuring the relative abundance of selected spots expressed as normalized %Vol in triplicate experiments.

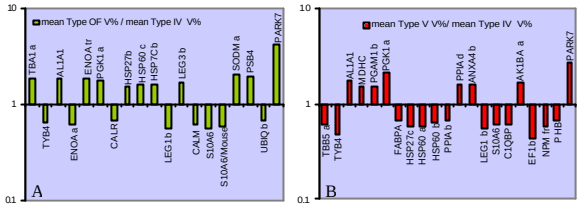

Fig3: Graphs illustrate protein modulation induced by OF/LB (A) and type V (B) substrates respectively, versus type IV collagen.

Interestingly, among the modulated proteins, we observed some heat shock proteins (HSP27, HSP60, HSP7C), tubulin alpha-1, one isoform of galectin, and proteasome subunit B4 (PSB4) which appear selectively enhanced in cell grown on OF/LB collagen *vs* type IV collagen, contrary to the cells grown on type V.
In conclusion, present date confirm and extend our previous data, supporting the hypothesis that different collagen substrates radiate influences which can modify, at least in part, the behaviour of neoplastic cells overlaying on them.

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LDL MODULATION OF HYALURONIC ACID SECRETION BY AORTIC SMOOTH MUSCLE CELLS

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Atherosclerosis is a chronic degenerative disease involving multiple processes and several cell types and molecules of the vascular wall. According to the response-to-retention hypothesis, the crucial event in atherosclerosis initiation is the retention and accumulation of low density lipoproteins (LDL) within the arterial wall, due to a combination of proteoglycan binding and LDL aggregation. This phenomenon triggers a cascade of events, such as LDL oxidation and fusion, ultimately leading to foam cell formation and lipid deposition (1). Such process causes local responses ending in the production of atherosclerotic plaques, which are formed by matrix proteins and glycosaminoglycans (GAG) like hyaluronic acid (HA). HA begins to be synthesized not only by the adventitia but also by 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 (2). SMCs are essential in neointima formation because of their ability to migrate and proliferate in response to different stimuli, like HA which they themselves secrete (3). The regulation of HA production by SMCs is expected to be one of the crucial steps for the atherosclerotic plaque formation and development; nevertheless the exact mechanism by which LDL particles modulate HA synthesis is not yet known.

In this work we focused on the role of different types of LDL (normal, oxidized, aggregated) on the production of HA and other GAG by aortic smooth muscle cells (AoSMC) which are one of the targets of LDLs in the intima. All these LDL particles are found in the circulation during different steps of atherogenesis development.

Native LDL were isolated from plasma of healthy donors by ultracentrifugation. LDL were then subjected to modifications such as oxidation and aggregation and labelled with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine) for fluorescence microscopy. The different LDL preparations show different mobility on agarose gel electrophoresis and are all internalized by the AoSMC as shown by fluorescence microscopy.

LDL particles influence the proliferation of cultured AoSMC in a dose-dependent manner. When added to the culture medium, LDL are able to increase the production of HA secreted into the medium as shown by HPLC analysis, with the greatest effect obtained by the oxidized LDL. Moreover, the production of pericellular HA is also increased as it can be visualized by particles exclusion assay. This HA increment seems to be due to an up-regulation of the hyaluronan synthase genes (Has2 and Has3) as shown by the real time PCR.

The fine definition of HA synthesis throws new insights on the knowledge of atherogenic progression, especially on the role of AoSMC in the early stages of this disease.

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ENERGY STATUS OF THE CELL REGULATES HYALURONAN SYNTHESIS

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Vascular pathologies are among the most common cause of death in western countries. Smooth muscle cells (SMC) have a critical role to onset arterial wall thickening though their proliferation, migration and secretion of extracellular matrix molecules and remodelling enzymes (1). Hyaluronan (HA) content is dramatically increased during neointima formation determining the reduction of vessel lumen.

(HA) is a linear, unsulfated glycosaminoglycan (GAG) that is composed of repeating units of D-glucuronic acid and *N*-acetylglucosamine (2). In mammals, three specific HA synthases (HAS1, -2, and -3) and three hyaluronidases (HYAL1, 2, and PH20) regulate HA synthesis and degradation. Contrary to other GAG, HA synthesis is on the plasma membrane.

At vascular level, HA increases SMC proliferation and migration inducing neointima formation (3). As its negative effects, HA synthesis inhibition can be a new strategy to prevent vessel thickening. Unfortunately, little is known on HA synthesis regulation. Recently, we discovered that HA synthesis strongly depends on the cytoplasmic concentration of its precursor UDP-glucuronic acid (UDP-GlcUA) (4).

As the synthesis of HA UDP-sugar precursors requires energy (i.e., ATP), we wonder whether the energy charge of the cells can regulate HA synthesis. As the main cellular sensor of ATP:AMP ratio is adenosine monophosphate activated protein kinase (AMPK) (5), we have assayed whether AMPK can control HA secretion and HAS activity in human aortic SMC (AoSMC).

AMPK is a ser/thr kinase which works as an energy sensor in all eukaryotic cells. In condition of low ATP content, this enzyme is phosphorylated and it is able to switch off several anabolic processes and switch on some catabolic pathways. Moreover AMPK activation can also be regulated by physiological stimuli, independent of the energy status of the cell, including hormones, nutrient depletion (i.e., 2-deoxyglucose, 2DG), and some drugs as metformin and AICAR.

To verify whether AMPK is involved in the regulation of HA metabolism, we treated AoSMC with various concentrations of AICAR (5), metformin and 2DG, and found a clear dose-response inhibition of HA synthesis. Interestingly, other GAG synthesis was not affected by the treatments. The involvement of AMPK in HA synthesis control was demonstrated by using mouse embryonic fibroblast from AMPK knock-out mice and by transfecting a dominant negative AMPK (DN-AMPK) and a constitutive active AMPK (CA-AMPK) expressing vectors in AoSMC.

Under a physiological point of view, we found that the activation of AMPK in AoSMC reduced proliferation, and migration suggesting that such treatment can prevent neointima formation *in vivo* (5).

Under a molecular point of view, we found that AMPK could inhibit HAS2 activity probably though phosphorylation. In fact, we overexpressed by transient transfection HAS2 and a constitutive active form of AMPK (CA-AMPK) in COS7 cells. Non transfected COS7 were not able to synthesize HA and we were not able to detect HAS activity in membrane preparations, whereas HAS2 overexpressing cells produced a large amount of HA and showed a high HAS activity. Interestingly, co-transfections with HAS2 and CA-AMPK completely inhibited HA synthesis as well as the HAS activity, confirming that AMPK controls HAS2 activity.

Such results indicate that the energy status of the cells can regulate HA synthesis opening new pharmacological strategies to inhibit HA synthesis to obtain vasoprotective effects.

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MITOCHONDRIOTROPIC RESVERATROL DERIVATIVES CYTOTOXIC TO FAST-GROWING CELLS

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Natural polyphenols are the object of intense interest because they display, at least in vitro, biomedically relevant activities. These effects are ascribed to their redox properties and to interactions with signaling proteins. Polyphenols can act either as anti- or pro-oxidants, that is, inhibitors or enhancers of oxidative and radical chain processes. This pro-oxidant or anti-oxidant activity may lead to useful oncological applications. Reactive Oxygen Species (ROS) are thought to be a major factor in cancerogenesis, and their production by mitochondria has a key role (1). Cancer cells are constitutively under oxidative stress and an intensification of this stress may lead to their selective elimination.

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene), the lead polyphenol-structure selected for this work, in addition to other important activities, has been reported to exert anti-proliferative and pro-apoptotic effects on various tumor-derived cells. Recently, it has been demonstrated that resveratrol-induced death of cultured mouse colorectal carcinoma cells (C-26) involves generation of superoxide anions – pro-oxidant action – by mitochondria. The IC_{50} for death induction was found to be in the hundreds of μM range, a concentration that cannot be reached in vivo due to the poor bioavailability of polyphenols (2).

Here, we report chemical modification of resveratrol aimed at targeting this polyphenol inside mitochondria. For this purpose, a membrane-permeable lipophilic triphenylphosphonium cation (3) has been linked to resveratrol. The new compounds, (4-triphenylphosphoniumbutyl)-4'-*O*-resveratrol iodide and its bis-acetylated derivative, accumulate inside cells and concentrate into mitochondria, as expected, where the anti- or pro-oxidant properties are most needed to intervene on processes of physiological and pathological interest. These compounds – intended to provide transient protection against metabolic conjugation – resulted to be cytotoxic for mouse colon carcinoma cells (C-26) and fast-growing but not slower-growing embryonic fibroblast (MEF) (4). Thus, they could represent a powerful potential tool to intervene on mitochondrial and cellular redox processes of pathophysiological relevance.

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MULTIPLE MYELOMA CELL AGGRESSIVENESS IS RESTRAINED BY HYPERFORIN

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Hyperforin (Hyp) is a natural phloroglucinol extracted from *Hypericum perforatum* (St John's wort), well known for its antidepressant activity. It has been found to possess several other biological properties (1), including promotion of apoptosis in leukemic B cells (2), and inhibitory effects on tumor invasion and angiogenesis (3). Multiple myeloma (MM) is an incurable B-cell malignancy characterized by the clonal expansion in the bone marrow of malignant plasma cells.

Here we evaluated whether Hyp has a role in promoting apoptosis of malignant plasma cells (PCs), and on their migratory and potential invasive capability. Flow cytometry analysis of six MM cell lines, and highly purified malignant PCs from eight patients with MM showed that Hyp induced apoptosis, as shown by dose-dependent stimulation of phosphatidylserine externalization and DNA fragmentation, by disruption of the mitochondrial trans-membrane potential, and cleavage of the caspase substrate PARP-1.

In addition, Hyp downregulated CXCR4 expression on malignant PCs, and restrained their migratory capability towards CXCL12, a CXCR4 specific ligand (4, 5). Finally, treatment of MM cells with Hyp resulted in a marked inhibition of their capacity to secrete matrix metalloproteinase-9, which is instrumental in degradation of extracellular matrix components taking place in angiogenesis and cancer invasion. All these effects were dose-dependent and in the µM range measured in the blood of people under *Hypericum* extract treatment.

Altogether, these properties qualify Hyp as new efficacious natural drug in the treatment of various diseases, including some haematological malignant tumors such as MM.

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