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# *European Journal of Histochemistry*

## *a journal of functional cytology*

The *European Journal of Histochemistry* was founded in 1954 by Maffo Vialli and published till 1979 under the title of *Rivista di Istochimica Normale e Patologica*, from 1980 to 1990 as *Basic and Applied Histochemistry* and in 1991 as *European Journal of Basic and Applied Histochemistry*. It is now published under the auspices of the University of Pavia, Italy.

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## NOVEL ASPECTS IN MATRIX PATHOBIOLOGY: ASSEMBLY, SIGNALING AND MOLECULAR TARGETING

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Extracellular matrices (ECMs) are highly dynamic and specialized three-dimensional (3D) scaffolds into which cells reside in tissues. ECM exerts diverse roles, either providing tissues with structural integrity and mechanical properties essential for tissue functions or regulating cell phenotype and functions to maintain tissue homeostasis. ECM is composed of a variety of structural and effective macromolecules. These macromolecular components are interconnected forming complex networks that actively communicate with cells through binding to cellular receptors and matrix effectors. Deregulation of ECM is associated with the development and progression of several pathologic conditions. The coordinated actions of estrogen receptors (ERs) and matrix macromolecules are principal mediators of ECM remodelling in the tumor and the adjacent stroma. In breast cancer, ERs are critical biomarkers as their expression in breast tumor determines the disease-free survival yet guiding treatment decisions and predicting prognosis as well as response to endocrine therapy. We have previously established the regulatory role of ER $\beta$  in cell behaviour and ECM composition of the highly aggressive MDA-MB-231 cells. The dynamic interactions among ERs and major ECM macromolecules and effectors, such as growth factor receptors, proteoglycans and matrix metalloproteinases, affect cancer progression, cancer cell functional properties, epithelial-to-mesenchymal transition and epigenetics. In recent years, research is focused on the epigenetic regulation of ECM components, which is critical for the regulation of several cell functions including proliferation, migration, differentiation, and survival, as well as ECM remodelling. The dynamic interplay between microRNAs (miRNAs), ECM macromolecules, and the tumor microenvironment affects many aspects of human diseases. Circulating and secreted miRNAs, *via* membrane vesicles, affect cell-cell communication and cellular metabolic pathways. Current research is focusing on the miRNA-mediated ECM reorganization and their functional relationship in breast cancer cell lines with different ER $\alpha/\beta$  status, and how matrix-mediated miRNAs affect tumor progression.

## SIRTIIN 1 AS A NEW REGULATOR OF HAS2 EXPRESSION AND HA SYNTHESIS IN HUMAN VASCULAR SMOOTH MUSCLE CELLS

A. Parnigoni, I. Caon, P. Moretto, E. Caravà, M. Viola, E. Karousou, A. Passi, D. Vigetti

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An altered balance between deposition and degradation of extracellular matrix (ECM) components within vessel walls, is a critical event in the development of cardiovascular diseases, such as atherosclerosis and restenosis.<sup>1</sup> Hyaluronan (HA) is one of the most abundant glycosaminoglycan in vascular smooth muscle cells (SMC). Interestingly, an abnormal accumulation of HA within vessel walls is often associated to tissue inflammation, as

HA can modulate SMC motility and differentiation, stimulates the synthesis of pro-inflammatory mediators, LDL adhesion and vessel thickening.<sup>2,3,4</sup> Among the three transmembrane isoenzymes involved in HA synthesis, hyaluronan synthase 2 (HAS2) plays the major role, using cytosolic UDP glucuronic acid (GlcA) and UDP N-acetyl glucosamine (GlcNAc) as substrates<sup>5</sup>. The synthesis of UDP-GlcA, through the activity of UDP-glucose 6-dehydrogenase (UGDH) enzyme, can alter NAD<sup>+</sup>/NADH ratio, as 2 molecules of NAD<sup>+</sup> are required to convert UDP glucose to UDP-GlcA. NAD<sup>+</sup>/NADH ratio is an important indicator of the energetic status of the cells<sup>6</sup> and it is crucial to stimulate the NAD<sup>+</sup> dependent deacetylase activity of sirtuins.<sup>7</sup> Our results show that HAS2 expression can be modulated by the master metabolic sensor sirtuin 1 (SIRT1), which exerts a positive function in vascular pathologies, protecting from DNA damages and inhibiting atherosclerosis and neointima formation.<sup>8</sup> The treatment with SIRT1 activators (SRT1720 and resveratrol) decreased HAS2 expression and reduced HA accumulation in the pericellular coat of SMC. Such treatments were able to revert the pro-inflammatory effects of TNF $\alpha$ , reducing HA-mediated monocyte adhesion and inhibiting SMC migration. At mechanistic level, we found that SIRT1 activation inhibited NF-kB/p65 nuclear translocation and decreased the levels of HAS2-AS1, a long-non coding RNA that epigenetically controls HAS2 mRNA expression, demonstrating for the first time that SIRT1 can prevent inflammation through inhibition of HA metabolism.

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## FILOPODIA AND TUNNELING NANOTUBES IN 3D CULTURES OF BREAST CANCER CELLS

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Cancer cells invasion into the surrounding extracellular matrix (ECM) takes place when cell-cell junctions are disrupted upon epithelial-mesenchymal transition (EMT). Both tumor-stroma and also tumor-tumor crosstalk *via* secretion of soluble factors are essential to support continuous tumor invasion. However, cancer cells also release macrovesicles and exosomes (containing bioactive molecules and signal peptides) which are taken up by neighboring cells or carried to distant sites, thus supporting intercellular communication and cargo transfer. Besides this indirect communication mode, cancer cells can develop cytoplasmic intercellular protrusions or tunneling nanotubes (TNTs) which allow for a direct communication and molecular exchange between connected distant cells.<sup>1-3</sup> In this study we described for the first time at the scanning electron microscope (SEM) TNTs

and very long flexible filopodia which developed from very aggressive MDA-MB-231 and low invasive shER $\beta$  MDA-Mb-231 breast cancer cells in three-dimensional (3D) cultures with different substrates (fibronectin, collagen), both with and without estradiol-2 (E2) treatment. In general, E2 seemed to highly affect breast cancer cells by favoring both long filopodia and TNTs growth: in particular, the low aggressive shER $\beta$  MDA-Mb-231 breast cancer cells treated with E2 in 3D collagen matrix showed the highest development of TNTs and filopodia. TNTs were often associated to adhering exosomes and microvesicles probably surfing from one cell to another, but no filopodia exhibited like-vesicles cytoplasmic structures on their surface. Ultrastructural investigations suggest that these long filopodia have only a mechanical and sensory role on the 3D collagen substrate but do not contribute to intercellular connections. Moreover, some of TNTs which showed a larger diameter appeared to be composed of a dozen individual TNTs, grouped together in a spiral array to probably ensure further resistance to intercellular communications when cancer cells move away one from each other during invasion of ECM. The same cells cultivated in 2D cultures on flasks did not exhibit long filopodia or TNTs in 2D cultures, thus confirming that 3D cultures in substrates mimicking the *in vivo* microenvironment are an essential model to investigate cancer cell invasion.

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### 6-MER HYALURONAN OLIGOSACCHARIDES MODULATE INFLAMMATION AND THYROID SPECIFIC GENE EXPRESSION IN HUMAN THYROCYTES BY UP-REGULATING TLR-4 AND CD44 RECEPTORS

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Grave's diseases and Hashimoto thyroiditis are autoimmune diseases associated to immune system activation that result in inflammation and altered thyroid function. In particular, it has been reported a decreased expression in thyroglobulin (Tg) and sodium iodide symporter (NIS) and an increase in hyaluronan (HA) production that accumulates in the gland.<sup>1</sup> Small HA fragments produced during pathological state may induce an inflammatory response in a variety of tissues, especially by interacting with the toll-like receptor 4 (TLR-4) and CD44.<sup>2</sup> The receptor stimulation triggers different pathways that culminate with the activation of the transcriptional nuclear factor kappa B (NF- $\kappa$ B) that in turn modulates the expression of inflammation mediators. This study investigated the potential of HA fragments in IL-1 $\beta$ -induced inflammation in human thyrocytes. Primary thyrocytes were obtained from benign thyroid nodules. Cultured cells were treated for 24 h with IL-1 $\beta$  (5 ng/ml) alone and in combination with 6-mer HA oligosaccharides (50  $\mu$ g/ml). mRNA and related protein levels of TLR4, CD44, interleukin-6 (IL-6), tumor necrosis factor (TNF- $\alpha$ ), Tg and NIS were evaluated by real-time PCR, western blot and ELISA, respectively. The level of NF- $\kappa$ B activation was also assayed. IL-1 $\beta$  induced NF- $\kappa$ B activation

and up-regulation of IL-6 and TNF- $\alpha$ , while reduced Tg and NIS expression. Treatment of thyrocytes exposed to IL-1 $\beta$  with 6-mer HA synergistically modulated the same parameters affected by IL-1 $\beta$  and induced up-regulation of TLR-4 and CD44. The addition of specific TLR-4 and CD44 blocking antibodies and/or hyaluronan binding protein (HABP), which inhibits HA activity, in cells treated with both IL-1 $\beta$  and 6-mer HA, reduced NF- $\kappa$ B activation and pro-inflammatory mediator production, while partially restored Tg and NIS levels. These findings suggest that small HA fragments may amplify the IL-1 $\beta$ -induced inflammation in thyrocytes *via* TLR-4 and CD44, concurring to decrease the expression of specific thyroid genes that play an essential role in gland function. Therefore, in autoimmune thyroid diseases the accumulation of HA could contribute to the inflammatory process and organ damage.

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### A POSSIBLE ROLE OF ENDOCAN IN CARTILAGE JOINT DISEASES

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Endocan or Endothelial Specific Molecule-1 (ESM-1) is a small soluble proteoglycan (PG), which plays important roles in immunity, inflammation and endothelial function.<sup>1</sup> Increased levels of such PG in peripheral circulation is considered as an indicator of angiogenesis and endothelial cells activation.<sup>2</sup> Endocan expression has been associated with a growing number of pathological conditions where chronic inflammation is a leading cause of disease as well as in highly vascularized cancers.<sup>1,3</sup> Its expression could be up-regulated by inflammatory cytokines and proangiogenic molecules in a NF- $\kappa$ B dependent manner.<sup>2</sup> The aim of this study was to evaluate endocan production in human chondrocytes during inflammation mediated by IL-1 $\beta$  administration. The degree of NF- $\kappa$ B activation and inflammatory cytokines production were also evaluated. We found that endocan is significantly up-regulated in IL-1 $\beta$  activated chondrocytes as well as NF- $\kappa$ B activity and the inflammatory parameters. On the contrary, by using a specific NF- $\kappa$ B inhibitor, before IL-1 $\beta$  administration we observed a significant decrease in endocan expression and inflammatory parameters. As reported endocan is significantly increased in arthritic tissues.<sup>4</sup> The results of our study indicated that endocan is also expressed in human chondrocytes; furthermore, consistent with previous results in other cell types and tissues, endocan increased expression is part of IL-1 $\beta$  induced inflammation and NF- $\kappa$ B activation. Further studies are needed to confirm the relevance of endocan in cartilage joint diseases.

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## ROLE OF HEPARANASE IN TUMOR DEVELOPMENT AND PROGRESSION

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The ability to invade tissues and to metastasize is one of the fundamental characteristics that make malignant tumor cells so difficult to eliminate surgically and with anti-cancer therapy. Tumor-stroma interactions and, more generally with the extracellular matrix (ECM), allow malignant cells to survive and promote metastasis.<sup>1</sup> The synthesis and remodeling of PG and GAG are deregulated during tumor progression with changes in their expression and turn-over, in post-translational modifications, as well as for an increase in the expression and activity of endoglycosidases.<sup>2</sup> Among endoglycosidases, heparanase (HPSE) has proved to be particularly crucial in cancer progression and metastasis. This enzyme, which cuts the heparan sulfate (HS) chains of proteoglycans, is overexpressed in several if not all types of primary human tumors, thus providing strong evidence of its pro-invasive and pro-angiogenic characteristics.<sup>3</sup> Furthermore, it has been demonstrated that heparanase promotes cancer progression by facilitating the release of many HS-related molecules such as growth factors, cytokines and enzymes. In more recent times, the range of possible roles played by heparanase has further expanded, including the regulation of gene transcription, the formation and release of exosomes, the activation of various signaling pathways and of autophagy, the promotion of coagulation cascade and chemoresistance as well as the conditioning of the tumor micro-environment.<sup>4</sup> Since it has been shown that treatment of neoplasms with compounds capable of inhibiting heparanase activity significantly attenuates tumor progression in different animal models of tumorigenesis, it can be hypothesized that an association of anti-heparanase therapy with anti-tumor conventional drugs could be the path to be followed in the near future.

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## HEPARANASE INVOLVEMENT IN EMT AND CANCER STEM CELLS OF PROSTATE TUMOR

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Prostate cancer display a certain phenotypic plasticity that allows the transition of cells from the epithelial to mesenchymal state. This process known as epithelial-mesenchymal transition (EMT) is one of the factors that gives the tumor greater invasive and migratory capacity with subsequent formation of metastases. In addition, scientific literature has shown that many cancers, including prostate cancer, are derived from a cell population that shows the properties of stem cells. These cells, called cancer stem cells (CSCs) or tumor-initiating cells, not only initiate the

tumor process and growth but are also able to mediate metastasis and drug resistance. EMT is also linked to stem-cell signatures in cancer cells. However, the impact of EMT and CSCs in prostate cancer progression and patient survival is still far from fully understood. Heparanase (HPSE) is the sole mammalian endoglycosidase capable of degrading heparan sulphate (HS).<sup>1</sup> HPSE is also involved in prostate cancer progression.<sup>2</sup> We previously proved that HPSE regulates EMT in non-cancerous pathologies.<sup>3</sup> Two prostate cancer cell lines (LNCaP and PC3) were silenced and overexpressed for HPSE. Expression of EMT and stemness markers was evaluated. Results showed that the expression of several EMT markers are modified by HPSE expression in both the prostate cancer cell lines analysed. In the same way, the stemness markers and features are also modulated by HPSE expression. Taken together, the present findings seem to prove a new mechanism of action of HPSE in sustaining prostate cancer growth and diffusion. As for other tumors, these results highlight the importance of HPSE as a potential pharmacological target in prostate cancer treatment.

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## STEERING CELL ACTIVITY THROUGH BIOMATERIALS AND SCAFFOLDS DESIGN

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A key factor in scaffold-based tissue and organ regeneration relies on enhancing (stem) cell-material interactions to obtain the same original functionality. Different approaches comprise delivery of biological factors and surface topography modifications. Although both strategies have demonstrated to amplify cell activity on biomaterials, they are still characterized by limited control in space and time, which may impede the proper regeneration of complex tissues. Here, we present a few examples where the converging biofabrication technologies allowed for the generation of a new library of 3D scaffolds with tailored biological, physical, and chemical cues at multiple scales. By engineering their topological properties, these porous biomaterials influence the activity of seeded cells, thereby initiating the regeneration of targeted tissues. Future efforts should aim at further improving our understanding of scaffold topological properties to achieve a fine control on cell fate. This will enable the regeneration of complex tissues including vasculature and innervation, which will result in enhanced *in vivo* integration with surrounding tissues. By doing so, the gap from tissue to organ regeneration will be reduced, bringing regenerative medicine technologies closer to the clinics.



## AN ELECTROSPUN POLYCAPROLACTONE SCAFFOLD FUNCTIONALIZED WITH GLYCOSAMINOGLYCANS FOR PERIPHERAL NERVE REGENERATION

A.J. Lepedda<sup>1</sup>, P. Wieringa<sup>2</sup>, M. Idini<sup>1</sup>, G. Nieddu<sup>1</sup>, S. Rocchiccioli<sup>3</sup>, N. Ucciferri<sup>3</sup>, M. Formato<sup>1</sup>, L. Moroni<sup>2</sup>

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When a peripheral nerve undergoes a traumatic injury, changes at the site of injury begin almost immediately and the healing process is guided primarily by glial cells called Schwann cells, which have the potential to regenerate a mature nerve. However, when the gap between proximal and distal segments is too large to allow for a complete self-regeneration, microsurgical intervention is essential for nerve repair.<sup>1</sup> In this respect, our aim was to develop a synthetic scaffold with both mechanical and biochemical properties that could guide Schwann cells in this process.<sup>2</sup> We fabricate fibrous polycaprolactone scaffolds with both random and aligned fibres functionalized with glycosaminoglycans (GAGs) purified from porcine aortic root. Neuronal Schwann cells RT4-D6P2T were assessed for proliferation, metabolic activity, and GAGs neo-synthesis during 7 days of culture. The expression of specific markers, *i.e.* Syndecan 1, Syndecan 4, Integrin, Laminin and p75, was evaluated by both immunofluorescence and western blot analyses. Furthermore, LC-MSMS-based proteomics was applied to gain a complete protein profile of Schwann cells as well as to identify differentially expressed proteins between cells grown on different constructs. Two well-defined fiber deposition patterns were successfully functionalized with GAGs. Both aligned and random fiber scaffolds functionalized with GAGs resulted in increased cell proliferation at day 1. In addition, aligned functionalized scaffolds also resulted in increased GAGs synthesis at day 1, probably due to cell extracellular matrix formation, and increased Syndecan-4 expression at day 7. Furthermore, specific pattern of protein expression was evidenced in presence of GAGs with respect to no-GAG scaffolds by differential proteomic analysis. In conclusion, we have shown that GAG-functionalized scaffolds are effective in modulating Schwann cell behaviour in terms of adhesion, proliferation, and differentiation and should be considered in strategies to improve PNS repair.<sup>3</sup>

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## DO GROWTH PLATE CARTILAGE CHONDROCYTES UNDERGO APOPTOSIS OR TRANS-DIFFERENTIATION INTO OSTEOBLASTS? A MORPHOLOGICAL CONTRIBUTION TO THE QUESTION

U.E. Pazzaglia<sup>1</sup>, M. Reguzzoni<sup>2</sup>, L. Casati<sup>3</sup>, S. Bighetti<sup>2</sup>, M. Protasoni<sup>2</sup>, M. Raspanti<sup>2</sup>

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The question of the "fate" of hypertrophic chondrocytes in the

growth plate cartilage has been long debated and is still discussed today with the most recent ultrastructural data reporting that chondrocytes undergo to apoptosis and are then engulfed by chondroclasts,<sup>1-3</sup> while studies based on immune-cytochemical characterization<sup>4,5</sup> and genetic-lineage tracing techniques<sup>6,7</sup> support the hypertrophic chondrocyte survival and transformation into osteoblasts (trans-differentiation). The present study was carried out on the proximal tibia metaphysis of growing rabbits, where it was possible a precise, topographic correlation of histological and ultrastructural images for what concerned the chondrocyte maturative process, the intercolumnar septa mineralization, the vascular invasion and the endochondral ossification beginning. Light microscopy, SEM of undecalcified 7 µm thick sections and fractured hemi-surface of the metaphysic and TEM ultra-thin, decalcified sections were employed, which allowed a careful observation of the cells present at different levels of the growth plate and a semi-quantitative evaluation of their distribution. No hypertrophic chondrocyte could be observed below the line of vascular invasion, where only globular masses of an amorphous, necrotic material (HC ghosts) surrounded by a large number of macrophages and other blood cell precursors could be recorded. The capillary advancement between the intercolumnar septa associated with osteoblast lined on the calcified cartilage columns was observed, but never an evidence of transformation (as the size reduction) of voluminous cells such as hypertrophic chondrocytes. In conclusion, on a morphological and morphometric basis the different cell types present below the vascular invasion line were consistent with a committed function for each of them in the general layout of the growth plate, not confirming the hypothesis of chondrocyte-osteoblasts trans-differentiation.

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## ENZYMATIC BIOSENSORS FOR BIOCHEMICAL MONITORING OF THE EXTRACELLULAR MATRIX: CHARACTERISTICS, INTERFERENCES AND STRATEGIES FOR SAFEGUARDING ANALYTICAL PERFORMANCES

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A biosensor is defined as "a self-contained analytical device that combines a biological component with a physicochemical device for the detection of an analyte of biological importance".<sup>1</sup> This specialized device is based on the recognition due to the presence of a biological element able to selectively recognize and transform the analyte under study in a detectable byproduct.<sup>2</sup> Several biological elements are used in biosensing: among them nucleic acids, receptors, antibodies, whole cells and much more frequently different class of enzymes, that are specific for the studied compound and must remain stable under normal conditions of use and storage. The biosensor design is conceived dependently on the analyte, so the working electrode (WE) materials and the components layered onto the WE surface<sup>3</sup> are very important.



Equally important is the matrix in which the analyte is to be measured; this is particularly true for implantable biosensors. Actually, enzyme biosensors are particularly prone to the interferences derived from different compounds present in the sample matrix. This problem became particularly problematic in biological samples (as fluids or tissues for instance), especially when electrochemical biosensors are used.<sup>4</sup> In fact, in this kind of matrix, numerous molecules as cells, proteins, and small oxidizable metabolites can interfere with the analyte detection. The biosensor performance can also be affected by other phenomena such as inflammations, neoplasias or other pathological conditions, as they can modify some chemical parameters as pH or fluids' composition which, in fact, may interfere with the enzymatic activity.<sup>2</sup> Sample manipulations, as derivatization, concentration, extraction or pre-filtration, can be useful in order to avoid matrix interferences,<sup>5</sup> although the direct usage of biosensors in a matrix is more desirable because it avoids the sample manipulations and eventual loss of analyte.

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### SMAD SIGNALLING PATHWAYS IN DERMAL CALCIFICATION

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Vascular mineralization is frequently observed in aging tissues as an active and progressive process occurring in several conditions as atherosclerosis and chronic kidney disease.<sup>1</sup> By contrast, dermal calcification is a rare event during aging, although it represents a typical finding in calciphylaxis and in a number of hormonal and genetic diseases. Calciphylaxis causes blood clots, painful skin ulcers and may cause serious infections possibly leading to death, whereas some hormonal disorders responsible for unbalanced ratio of circulating levels of calcium and phosphate and genetic diseases, characterized by ectopic calcification, may involve altered expression of pro- and/or anti-mineralizing factors.<sup>2</sup> In the last years, it has been highlighted the role of specific signaling pathways in the development of aberrant mineral precipitation in the vascular system, but few data are available concerning dermal pathologic mineralization in genetic disorders. Therefore, aim of this study was to investigate Smad signaling pathways, *i.e.* phosphorylated Smad2/3 and Smad1/5/8, in the dermis of patients with soft connective tissue alterations, since these proteins are canonical mediators of TGF $\beta$  and BMP signalling, respectively.<sup>3</sup> Skin samples were taken from three control subjects undergoing surgical procedures and from five patients with mineralized phenotype who underwent histopathologic diagnosis on skin biopsies. Subsequent biomolecular analyses confirmed the genetic basis of ectopic calcification. The study was performed according to the principles of the Declaration of Helsinki. Samples have been routinely processed for ultrastructure and immunohistochemistry analyses. Electron microscopy confirmed the presence of mineralized elastic fibers mainly localized in the reticular dermis of patients. Immuno-his-

tochemistry revealed that mesenchymal cells present in pathological samples were strongly stained for both pSmad2/3 and pSmad1/5/8, whereas in control biopsies cells were weakly positive. To be noted that positive cells were spread through the whole dermal thickness. Results indicate that both TGF- $\beta$  and BMP signalling pathways are activated thus contributing to dermal calcification in addition to local modulatory factors specifically favouring elastic fiber calcification.

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### BIO-INSPIRED ELECTROSPUN SCAFFOLDS IN TISSUE ENGINEERING

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Bio-inspired natural polymers as gelatin (GE), polylactic acid in its racemic form (PDLLA), and a human tropoelastin-derived peptide were herein successfully electrospun for producing scaffolds suitable for applications in tissue engineering. NanoCrystalline Cellulose (NCC) was added to the polymeric mixture and the cross-linking reaction was also carried out in order to confer them strength and insolubility in aqueous media, respectively. The morphology of the scaffolds was investigated by Scanning Electron Microscopy (SEM). The results show that the cross-linking did not alter the three-dimensional fibrillar microstructure characterized by the absence of beads and the presence of interconnected pores, as well. Furthermore, the diameters of the fibers in electrospun scaffolds were tunable as a function of polymeric mixture and concentration. Swelling test and contact angle measurements demonstrated that the hydrophilicity increased after the addition of NCC and human tropoelastin peptide. The tensile properties of the scaffolds were also evaluated through E-modulus and ultimate tensile strength (UTS) showing a significant decrease after the swelling test.

### N-ACETYL-CYSTEINE AMELIORATES POST-NATAL SKELETAL GROWTH IN AN ANIMAL MODEL OF DIASTROPHIC DYSPLASIA

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Diastrophic dysplasia is a recessive chondrodysplasia caused by mutations in the *SLC26A2* gene encoding for a sulfate/chloride antiporter of the cell membrane. Functional impairment of the

transporter leads to reduced cytosolic sulfate level causing cartilage proteoglycan (PG) undersulfation. Intracellular sulfate is mainly dependent on extracellular uptake; in physiologic conditions a small amount comes from the catabolism of sulfur-containing amino acids and other thiols. This observation suggests that intracellular sulfate level might be increased through the catabolism of thiol compounds in order to improve PG sulfation. For this reason, we have investigated in dtd mice whether the treatment after birth with a cysteine derivative, N-acetylcysteine (NAC), might ameliorate the skeletal phenotype both at the biochemical and morphological level. 250 mg NAC/Kg body weight were administered to animals by hypodermic injections twice a day during the first week of life. At the end of the treatment, chondroitin sulfate HPLC disaccharide analysis showed a significant increase of PG sulfation in treated dtd mice compared to the placebo dtd group, while morphometric measurements of long bones did not show any significant difference. Therefore, a longer NAC treatment over the first 21 days of life was considered. In treated dtd mice a significant increase in PG sulfation was observed (84% vs 79% sulfated disaccharides in treated dtd vs placebo dtd group,  $P < 0.01$ ). The length of different skeletal elements (tibia, femur, radius, vertebrae and hip) was increased. Histology of the growth plate showed an amelioration of its architecture in NAC treated dtd mice compared to untreated animals further suggesting an amelioration of the endochondral ossification process. The improvement of the bone phenotype of NAC treated dtd mice was further demonstrated by an increase of the bone mineral content by DEXA (3.55 vs 1.25 mg in treated dtd vs placebo dtd group,  $P < 0.05$ ). Overall, our results demonstrated that NAC might pave the way for a pharmacological treatment of diastrophic dysplasia taking advantage of a drug repositioning strategy.

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#### 4-PHENYLBUTYRATE RESCUES CELLULAR STRESS IN RECESSIVE OSTEOGENESIS IMPERFECTA

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Cartilage associated protein (CRTAP), prolyl 3-hydroxylase 1 (P3H1), and cyclophilin B (CyPB) are the components of the prolyl-3-hydroxylation complex responsible for the post translational modifications of collagen type I specific proline residues on C-3 in the endoplasmic reticulum. Mutations in these genes cause a delay in collagen folding and its overmodification and are responsible for the recessive Osteogenesis Imperfecta types VII, VIII, and IX, respectively. Our study dissects the effects of these defects on cellular homeostasis of primary fibroblasts from recessive OI patients. Cellular retention of overmodified collagen and presence of protein aggregates within the cells, promoting the activation of the PERK branch of unfolded protein response

(UPR) and impairing cell survival despite the autophagy upregulation, were demonstrated. Treatment of mutant cells with 4-phenylbutyrate (4-PBA) increased protein secretion, promoted autophagy and normalized UPR and apoptotic markers expression. To dissect whether the positive effect of 4-PBA was due to its autophagy stimulator ability or to its chaperone function, ER proteostasis, PERK activation and cell survival were monitored in absence or presence of a pharmacological inhibitor of autophagy. Interestingly, 4-PBA treatment, even when autophagy was impaired, partially rescued ER proteostasis, and restored pPERK levels and cell survival, supporting its major role of chaperone in ameliorating cell homeostasis. Our results demonstrated that the intracellular stress in recessive OI can be tuned by 4-PBA therapy and provide insight into the molecular mechanisms of 4-PBA action.

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#### AMYOTROPHIC LATERAL SCLEROSIS AND TDP-43: AN EPIGENETIC LIAISON

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Amyotrophic Lateral Sclerosis (ALS) is an adult-onset neurodegenerative disease, characterized by the progressive degeneration of upper and lower motor neurons. ALS is predominantly sporadic (sALS, 90% of cases), with no clear genetic linkage, and environmental triggers may be involved in disease initiation since environmental exposure to toxins, excessive physical activity, dietary factors, and changes in immunity increase the risk of developing sporadic ALS. These factors may drive epigenetic changes (including histone modifications, DNA methylation and RNA editing), which are well suited to explain disease onset and progression in ALS, as they may be acquired throughout life.<sup>1</sup> Many groups of epigenetic modifiers and consequently different epigenetic modifications have been linked to neurodegenerative processes, such as loss of normal heterochromatin (decrease in H3K9me2 and HP1 $\alpha$ ) that promotes tau-mediated neurodegeneration *in vivo*, and aberrant DNA methylation in animal and cellular models of ALS, obtained by the overexpression of one of the ALS-causing genes, identified in patients showing familial inheritance (fALS).<sup>2</sup> Among the ALS-causing genes, we focused our attention on TDP-43. TDP-43 has functions in transcription, RNA processing, microRNA biogenesis and RNA splicing. A small portion of TDP-43 is expressed in the cytosol, where it may be involved in stress granular formation and mRNA stability.<sup>3</sup> By using *in vivo* and *in vitro* models we performed a detailed analysis of histone modifications and DNA methylation in cells expressing mutant TDP-43 and most importantly we identified the histone deacetylase1 (HDAC1) as specific TDP-43 interactor.

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## TESTING THE EFFECTIVENESS OF THE ALZHEIMER'S DISEASE CURRENT THERAPIES IN *DROSOPHILA MELANOGASTER* TRANSGENIC MODEL

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Alzheimer's Disease (AD) is a progressive neurodegenerative disorder; it is characterized by short-term memory loss, shortened lifespan, locomotive defects and cognitive impairment.<sup>1</sup> AD currently affects 50 million people around the world. The disease was firstly characterized by Aloise Alzheimer in 1907 after an autopsy on a 55-years-old woman named Auguste Deter, who died with progressive cognitive disorder.<sup>2</sup> Abnormal intercellular aggregates and a special substance were found in her brain.<sup>2</sup> Nowadays, the Amyloid- $\beta$  plaques and the Neurofibrillary Tangles (NFTs) are recognized as AD pathological hallmarks.<sup>3-4</sup> The presence of A $\beta$ -plaques and NFTs lead to synaptic disruption, neuronal dystrophy and neuronal loss. In order to deliver the expression of the hyperphosphorylated Tau protein and A $\beta$ -peptides in the Central Nervous System (CNS), we have exploited the UAS/Gal4 System using wild type and transgenic flies. We have therefore modelled *Drosophila Melanogaster* to test the FDA approved therapies for AD. We used two different type of drugs for all the assay, an AcetylCholinesterase Inhibitor (AChEI) such as Donepezil, Rivastigmine and Galantamine, and an N-Methyl-D-Aspartate receptor antagonist (NMDAR antagonist) Memantine. Each drug was administered at fixed concentration. Donepezil showed an improvement of the symptoms for both transgenic lines except for the climbing assay in Tau line. Memantine ameliorated the lifespan and the A $\beta$ -deposition in the brain of the fruit flies in the A $\beta$ -Arctic line, but not in the Tau line. Further to this, Memantine has not rescued the induced locomotive defects of both transgenic lines, along with negative results for the Rough Eye Phenotype analysis. Galantamine failed to ameliorate the induced AD symptomatology on A $\beta$ -Arctic line while reduced the symptomatology and the immunostaining signal in the Tau line.

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## THE INVISIBLE AND NEGLECTED ASPECT THAT AFFECTS THE NEW GENERATION OF HEART VALVE BIOPROSTHESES: AN APPROACH TO ALPHA-GAL RELATED ISSUE SOLVING

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The most common heart valve disease in industrialized countries is Aortic Stenosis (AS). Unfortunately, more than 30% of patients suffering for AS are not eligible for the conventional surgical approach due to the high risk of mortality associated with

ageing or comorbidities.<sup>1</sup> A decade ago, Transcatheter Heart Valve Bioprotheses (THVBs) emerged as a valuable option for patients with inoperable high-risk severe symptomatic AS. The safety and effectiveness of THVBs are recently emerging by studies which prove satisfactory outcomes in high/middle-risk categories.<sup>2-4</sup> However, the animal tissue used for manufacturing THVBs is the same xenogeneic pericardium adopted for Surgical Bioprosthetic Heart Valves (SBHVs), so that the issue of the long-term substitute durability remains unresolved and of crucial importance. Calcification of SBHVs occurs 10/12 years after implantation, and only now it appears to affect THVBs since their clinical follow-up is still limited to a maximum of 8 years. At this time, THVBs suffer from a time-dependent degeneration issue consisting of endothelial hyperplasia, fibrosis and structured thrombus formation.<sup>5</sup> Therefore, since in a few years the dystrophic degeneration concern will also affect THVBs, it is important to combine the advantages of minimally-invasive access with an innovation capable of preventing or slowing the degenerative processes and increasing their lifespan. In particular it is mandatory to extend the bioprosthetic tissue biocompatibility by interfering with the standard treatment with glutaraldehyde to achieve a more effective inactivation of the xenoantigenic component. Recently, a treatment called FACTA was shown to be effective in tissues intended for bioprosthetic heart valves manufacture, by increasing resistance to enzymatic and oxidative degradation. Moreover, treated tissues showed a significant improvement in biocompatibility due to the almost complete inactivation of the xenogeneic alpha-Gal epitope, still present in significant amount in the current commercial SBHVs and THVBs.<sup>6</sup> From extensive *in vivo* studies in both swine and mice animal models, the treatment resulted to be effective in reducing thrombogenicity, inflammatory processes and bacterial adhesiveness. Lastly, as the general inactivation of the xenoantigenic component resulted to double the resistance to calcification, speculation is proposed on how FACTA can be an effective treatment to double the lifespan of a new generation of THVBs.

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## A NEW MEMBRANE REPAIR MECHANISM IN ERYTHROCYTES: ITS IMPLICATIONS IN CELLULAR AGING AND ITS ADAPTIVE CONTROL BY PRO-OXIDANT POLYMORPHISMS

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Erythrocytes undergo dramatic changes loosing approximately 30% of their membrane and Hb content at the end of their lifespan. We have demonstrated that the red cells possess a specific machinery aimed to concentrate the oxidized hemoglobin, that is formed during aging, in definite membrane areas that can be selectively removed leading to smaller but still viable cells.<sup>1</sup> Those areas are selectively phagocytized by



macrophages or lost in microvesicles that are subsequently trapped in the spleen. As a matter of facts, we have observed that in hereditary hemolytic diseases protecting against malaria, microvesicles accumulate in blood.<sup>2,3</sup> Malaria is a major environmental factor: more than half of human population is or was exposed to this disease. In endemic areas (including Sardinia) between 20% to 50% of the inhabitants acquired adaptive mechanisms that expose the red cells to a basal oxidant stress. We conducted a study in *P. falciparum* infected erythrocytes demonstrating that those mutations induce a rapid and selective destruction of the parasitized red cells triggering the same redox signaling response to that observed in erythrocyte senescence.<sup>4,5</sup> We are now collecting data from malaria patients (Quang Tri region, Vietnam) in order to validate the proposed mechanism with clinical data. Our study indicated that a re-modulation of a physiological mechanism is effective to kill life threatening malaria parasites acting on the host cells. In conclusion, the investigation of a complex physiological mechanism regulating the erythrocyte lifespan is helping the understanding of widespread adaptive mechanisms also revealing new pharmacological perspectives for malaria treatment.

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### **IN VITRO AND COMPUTATIONAL STUDIES OF SYK INHIBITORS WITH ANTIMALARIAL ACTIVITY IN RBCS, NEW PERSPECTIVES**

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Resistance to antimalarial drugs has spread rapidly over the past few decades, therefore, to overcome it, WHO recommends Artemisinin based combination therapies (ACTs) for the treatment against *Plasmodium falciparum* malaria. Although these treatments are effective in many parts of the world, there is serious concern that malaria parasites are once again developing widespread resistance to this vital treatment.<sup>1</sup> To date, artemisinin resistance has been confirmed in 5 countries of the Greater Mekong subregion. The development of resistance has highlighted the need for the search of novel antimalarial molecules. Recently, it has been demonstrated that Syk inhibitors represent a new class of antimalarial drugs that suppress merozoite egress<sup>2</sup> by inhibiting a host target that cannot be mutated by the parasite to develop drug resistance. Our purpose was to evaluate the antiplasmodial activity of different Syk inhibitors using both *in vitro* and computational screening methods and to have more information on the mechanisms responsible for the protein-ligand recognition and binding<sup>3</sup>. A detailed understanding of Syk Kinase/ligand interactions is, therefore, the central focus, in order to understanding biology at the molecular level, with the future perspectives of improving and modifying the compounds

structure and discover new class of drugs to obtain the highest efficacy of inhibition in infected RBCs. We demonstrate here that the tested compounds show a high specificity<sup>4,5</sup> for the protein target Syk and its strong inhibition confirmed by the decrease of Tyr phosphorylation levels.

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### **MORPHOLOGICAL AND BIOCHEMICAL TRAITS OF TARGET MARINE SPONGES**

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Sponges (Phylum Porifera) are the lowest Metazoa with basal body plan architecture *viz.* aquiferous system (apertures/canals/filtering chambers), skeletal network of organic (fibres/filaments) and inorganic (spicules/sand) structures, and independent/mobile cells in a glycoproteic amorphous jelly as extracellular matrix (ECM). Sponges are able to synthesize a wide array of molecules that are prototypes of homologs produced by higher animals. For this reason, these compounds have a huge therapeutic potential in the applied research ranging from antitumoral, antiviral, anti-inflammatory, and antibiotic activities to new biomaterials discovery for cell therapy, biomedicine, cosmetics, and food industry. The evolutionary success of sponges since Cambrian is mainly due to adaptive strategies aimed at high body plasticity performed through a few cell types, totipotent cells, absence of true tissues, and the appearance of ECM as a key evolutionary novelty. Sponges ECM resembles that of higher taxa, being composed of collagen, proteoglycans (PGs), and minor amounts of structural proteins and glycosaminoglycans (GAGs), but their structural features and biological roles are poorly known. Basic structural knowledge is pivotal to test species suitability for applied research, together with biomaterial availability from sustainable sources (USAMA® mariculture). To perform morphological and biochemical characterization our aim is to look for some Mediterranean target horny sponges as experimental model on the basis of their optimal regenerative properties, easy availability, and successful *in situ* culture. To understand morpho-functional and physiological performances, our priority is to detect, at the topographic level, histological traits and then characterize histochemical and biochemical patterns of target species. For this purpose, we applied staining and analytical methods to cultured sponge clones. Our results highlight that histological and biochemical traits of the ECM target sponges can be considered a prototype structure compatible with the use *e.g.* as a scaffold in tissue engineering.

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