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The *European Journal of Histochemistry* was founded in 1954 by Maffo Vialli and published until 1979 under the title of *Rivista di Istochimica Normale e Patologica*, from 1980 to 1990 as *Basic and Applied Histochemistry* and in 1991 as *European Journal of Basic and Applied Histochemistry*. It is published under the auspices of the University of Pavia, Italy.
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SKELETAL DISORDERS CAUSED BY DEFECTS IN THE SYNTHESIS OF SULFATED GLYCOSAMINOGLYCANS

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Skeletal dysplasias with multiple dislocations are a group of severe disorders characterized by dislocations of large joints, scoliosis, short stature and a variable combination of cleft palate, heart defects and intellectual disability. The majority of these rare disorders have been linked to pathogenic variants in genes encoding glycosyltransferases, sulfotransferases, epimerases or transporters, required for glycosaminoglycan (GAG) biosynthesis. Among this group of inborn errors of development we are studying, using *in vitro* and *in vivo* models, diastrophic dysplasia, chondrodysplasia gPAPP type and Desbuquois dysplasia type 1 (DBQD1). Regarding this latter disorder we have recently elucidated the crucial role of a ER/Golgi nucleotidase in the biosynthesis of GAGs. DBQD1 is caused by mutations in Calcium-Activated Nucleotidase 1 (*CANT1*) a nucleotidase that preferentially hydrolyses UDP. We have studied the role of CANT1 in PG synthesis in cartilage and chondrocytes from a *Cant1* knock-out mouse (*Cant1*$^{-/-}$ mouse) which reproduces the growth defects observed in DBQD1. PG synthesis was studied in rib knock-out chondrocytes; mutant cells showed GAG chains with reduced hydrodynamic size, GAG oversulfation, reduced PG synthesis and impaired secretion. This latter observation was confirmed by transmission electron microscopy of mutant vs. wild type cartilage showing the presence of dilated ER suggesting a role of CANT1 in protein secretion. However, protein retention in *Cant1*$^{-/-}$ cells does not cause ER stress and UPR activation since microarray analysis demonstrated no overexpression of genes related to this pathway. Moreover, protein level of BiP and ATF4 and expression of Xbp1 spliced form were normal in *Cant1*$^{-/-}$ cells compared to controls.

In conclusion, the pathogenic mechanism of DBQD1 includes deregulated chondrocyte performance due to defective intracellular proteoglycan synthesis and altered proteoglycan properties in the extracellular matrix. This study highlights that the understanding of the molecular pathogenesis involving GAG chains is essential for proteoglycan biology and to facilitate the development of therapeutics and design of new drugs for skeletal diseases.

Work supported by the European Community (FP7, "Sybil" project, grant n.602300).

HYALURONAN MODULATES THE EXPRESSION LEVELS OF LNCRNAS MALAT1 AND GAS5 IN LPS-INDUCED INFLAMMATION IN MOUSE CHONDROCYTES

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Rheumatoid arthritis is a disabling autoimmune disease, characterized by inflammation of synovial tissues and cartilage degradation. Long non-coding RNAs (lncRNAs), are functional RNAs that modulate gene expression through multiple mechanisms, such as epigenetics, alternative splicing, small RNA sponging and transcriptional and translational regulation. Recent studies have shown a dysregulation of lncRNAs in rheumatoid arthritis and others inflammatory diseases. In particular, evidences have revealed increased expression levels of Grow arrest-specific 5 (GAS5), which up-regulates the gene expression of several MMPs and stimulates apoptosis[1], and metastasis associated lung adenocarcinoma transcript 1 (MALAT1), that increases p38 MAPK and NF-kB activation[2]. Hyaluronan (HA) is a non-sulfated major glycosaminoglycan (GAG) of the ECM and may exert different action depending on its degree of polymerization. It was reported that high molecular weight HA reduces inflammation in several experimental *in vivo* and *in vitro* models, but the exact mechanism seems to be yet unclear[3]. In this study we aimed to investigate the influence of HA on the gene expression of lncRNA GAS5 and MALAT1 and related microRNAs, miR21 and miR 125b, in human chondrocytes stimulated with lipopolysaccharide (LPS). Cell viability and apoptosis were assessed using MTT assay and caspase-3 activity. The expression levels of GAS-5, MALAT1, miR21 and miR 125b were measured using RTqPCR. The protein level of interleukin 1β (IL 1β), IL 6 and NF-kB was assayed by ELISA kits. LPS treatment decreased cell viability, increased cell apoptosis and promoted NF-kB activation and the release of pro-inflammatory factors. Furthermore, the expression levels of GAS5 and MALAT1 was increased, while miR21 and miR 125b was decreased in LPS-stimulated chondrocytes. The addition of HA decreased cell apoptosis, NF-κB activation, pro-inflammatory mediators and GAS5 and MALAT1 expression, while partially restored miR21and miR 125b levels.

Together these results show that the HA may exert a protective effect also acting as a negative regulator of GAS5 and MALAT1, that in turn, modulating miR21 and miR 125b, could contribute to regulate cell survival and inflammatory response.

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HEPARANASE REGULATES THE DEVELOPMENT OF FIBROSIS AND RENAL INJURY AFTER ISCHEMIA/REPERFUSION

V. Masola[1,2], G. Bellin[1-3], G. Vischini[4], L. Dall'Olmo[5], S. Granata[1], G. Gambaro[2], A. Lupo[1], G. Zaza[1], M. Onisto[2]

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Renal ischemia/reperfusion (I/R) injury occurs in patients undergoing renal transplantation and with acute kidney injury (AKI). It is responsible for the development of chronic allograft dysfunction characterized by parenchymal alteration and fibrosis. Heparanase (HPSE), an endoglycosidase that regulate EMT and macrophages polarization, is an active player in the biological response triggered by ischemia/reperfusion (I/R) injury[1,2]. I/R was induced in vivo by clamping left renal artery for 30 min in wt C57BL/6J mice. Animals were daily treated or not with Roneparstat (an inhibitor of HPSE) and sacrificed after 8 weeks. HPSE, fibrosis, EMT-markers, inflammation and oxidative stress were evaluated by biomolecular and histological methodologies together with the evaluation of renal histology and measurement of plasmatic and urine parameters of renal function. 8 weeks after I/R HPSE was upregulated both in renal parenchyma and plasma whereas tissue specimens showed clear

evidences of renal injury and fibrosis (IF/TA). The inhibition of HPSE with Roneparstat restored histology and fibrosis level comparable with that of control. I/R injured mice showed a significant increase of the EMT, inflammation and oxidative stress markers but they were significantly reduced by the treatment with Roneparstat. Finally, the inhibition of HPSE in vivo almost restored renal function as measured by BUN, plasma creatinine and albuminuria.
The present study point out that HPSE is actively involved in the mechanisms that supervise the development of renal fibrosis arising as a consequence of the ischemia-reperfusion damage. In the transplanted organ HPSE inhibition would therefore constitute a new pharmacological strategy to reduce acute kidney injury and to prevent the chronic pro-fibrotic damages induced by I/R.

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HEPARANASE REGULATES EMT AND STEMNESS FEATURES OF PROSTATE CANCER CELLS

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EMT is a process by which epithelial cells lose adherence junctions and apical–basal polarity and acquire a mesenchymal phenotype with an enhanced motility[1]. EMT is thought to be activated in cancer cells, linked to their dissociation from the primary tumour and their intravasation into blood vessels[2]. However, the impact of the EMT in cancer progression and patient survival is still far from fully understood. Epithelial to mesenchymal transition is also linked with stem cell signatures in cancer cells[3]. Heparanase is the sole mammalian endoglycosidase capable of degrading glycosaminoglycan heparan sulphate (HS). The enzymatic cleavage of HS by HPSE results in ECM remodelling, as well as in the release of bioactive mediators, producing a rapid tissue response to local or systemic stimuli. These effects profoundly affect multiple pathophysiological processes such as tumour progression, inflammation and fibrosis[4]. HPSE is involved also in prostate cancer progression[5]. We have proved in-vitro and in-vivo that HPSE regulates renal EMT induced by FGF-2[6], TGF-beta[7] and hypoxia[8]. The aim of the present project was to understand how different factors (FGF-2, TGF-beta, EGF, and hypoxia) induce EMT in prostate cancer cells and how HPSE could regulate this process and whether HPSE may modulate cancer stem cell features. Two prostate cancer cell lines were silenced and overexpressed for HPSE. Cells were then treated with FGF-2, TGF-beta and exposed to hypoxia. Gene expression of EMT and stemness markers was evaluated by real time PCR. Results showed that the expression of several EMT markers such as alpha-SMA, Vimentin, Fibronectin, and the associated transcription factors Twist, Slug and Snail are modified by HPSE expression in both the prostate cancer cell lines analysed. Similarly, also the stemness markers SOX-2, OCT-4 are modulated by HPSE expression.
Taken together the present findings prove a new mechanism of action of HPSE in sustaining prostate cancer growth and diffusion and stress the importance of HPSE as pharmacological target in cancer treatment.

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MODULATION OF STRUCTURE AND FUNCTION IN ARTICULAR CARTILAGE

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Articular cartilage has been for a long time the subject of extensive research, mostly carried out with light microscopy and/or transmission electron microscopy. These techniques are, however, unable to depict adequately the articular surface, which is the only portion directly involved in the articular function and must resist compressive and gliding forces different from the underlying matrix. In this study fragments of human, porcine and bovine articular cartilage were briefly fixed in 1% Karnowski, dehydrated in graded ethanol and hexamethyldisilazane and observed by SEM and by AFM. Some fragments were fixed in Cupromeronic Blue (CB) or in Ruthenium Hexammine Trihydrate (RHT), dehydrated and observed as above. Other samples were fixed overnight in 1% Karnowski, treated for 5 days with 2N NaOH and dehydrated and observed as above. Finally, some samples were fixed as above, processed for conventional light microscopy and stained with haematoxylin-eosin, Cupromeronic Blue or Sirius Red. In all cases the free (synovial) surface of the articular cartilage appeared devoid of cells and made essentially of a thick layer of glycoconjugates. Treatment with concentrated NaOH completely removed the non-collagenic material and revealed a uniform population of very thin (approx. 15-20 nm) fibrils, with various orientations depending from the sampling site. With the same treatment the subsurface matrix down to the subchondral bone appeared very different, with normal chondrocytic lacunae and larger, randomly oriented, heterogeneous collagen fibrils with diameters up to 150 nm. It is possible that the articular surface may just represent a high-turnover layer, whose fibrils remain thin because continuously lost and renewed. On the other hand, several studies report the coexistence in cartilage of two distinct populations of fibrils: a first one with thin, uniform fibrils approximately 20 nm in diameter and made of collagen types XI and II in a fixed ratio of 1:6, the other made of larger fibrils of types II, IX and XI, with a heterogeneous diameter starting at 40 nm and reaching far larger values.
In the light of these data our results may suggest the existence of a distinct superficial layer with chemical, structural and functional properties distinct from the underlying matrix. The research is still underway.

METAPHYSEAL CARTILAGE GROWTH IN THE FETAL AND POSTNATAL AGE

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In this research the longitudinal and peripheral growth patterns were analyzed in human anlagen cartilage autopods (fetal stage 20-29 w) through morphometric assessment of chondrocyte parameters between the peripheral and central sectors of the transition zone defined by the primary ossification center and the epiphyseal basis. The initial purpose was to correlate the chondrocyte dynamics with the longitudinal and peripheral growth. Subsequently a comparison was also made between the corresponding sectors of post-natal cartilage (3-5 m) to investigate the opposite direction of fetal periosteal ossification versus the Lacroix bone bark. We have evaluated % lacunar area, % total matrix area, total lacunar density and average single lacunar area, with the aim to identify a discrepancy between the growth of the epiphyseal and diaphyseal circumference at various stages of development. The morphologic and morphometric data of this study are consistent with this mechanical model because each intra-lacunar chondrocyte duplication also doubles the cellular mass and the hypertrophic/swelling transformation increases the cell mass inside the single lacuna. Before the mineral deposition the expansion was limited by the collagen network that surrounds the chondrocyte, followed by the onset of a rigid calcified scaffold. In the comparison of the peripheral and central sectors of the transition zone both conditions were present for both the perichondral appositional pattern and the longitudinal one in the columns of chondrocytes becoming hypertrophic. In the postnatal developmental stage, the longitudinal growth pattern of the central sectors was maintained with a similar columnar organization of the fetal transition zone, whereas the perichondrium was replaced by a new framework (Lacroix bone bark). The peripheral growth was achieved with a different mechanism characterized by a pronounced bending of the chondrocyte columns below the perichondrial bark, whose radius gradually decreased from the peripheral to the central sectors.
In conclusion the modeling of cartilage has been modulated by several factors: the coupled cells of the central epiphysis suggested a slower duplication rate than the clusters in the basal layer of the epiphysis. The lacunar shape resulted from the combined action of duplication rate and volumetric changes. The external, 3D constraints exerted by the inter-territorial matrix when completed by the mineral deposition representing a further, epigenetic factor.

COLLAGEN ARRAY AFFECTS THE PHENOTYPE OF BREAST CANCER CELLS

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Collagen acts like a barrier limiting or also favoring tumor cell invasion in extracellular matrix, so that peri tumoral collagen fibre array is considered a marker of mammary carcinoma progression. The collagen barriers (basal membrane, sub basal reticular collagen and dermis collagen fibres) which breast cancer cells come across during invasion of stroma were reproduced in 3D cultures to study how different collagen substrates affect the phenotype of breast cancer cells (MCF-7 and MDA-MB-231). The 3D cultures on a) Millipore filter, b) Millipore filter+Matrigel, c) Millipore filter+type I collagen, d) collagen membrane with fibres parallel to the culture plane and e) collagen membrane with fibres perpendicular to the culture plane were analyzed at the scanning electron microscope. MCF-7 cells on Millipore filter and Millipore filter+Matrigel show a spherical shape and microvilli, whereas MDA-MB-31 cells show fusiform or spherical shapes with few microvilli but many microvesicles. Contact with type I reticular collagen fibrils induces an epithelial-mesenchymal transition in MCF-7 cells. MCF-7 and MDA-MB-231 cells cultivated on a collagen membrane of type I collagen fibres, parallel to the culture plane, show spherical shaped cells with an increase of microvilli and microvesicles respectively, but do not penetrate into the collagen membrane; however exosomes and microvesicles are present into the thickness of the collagen membrane only in MDA-MB-231 cell cultures. MCF-7 cells grown on a collagen membrane of type I collagen fibres, orthogonally arranged to the culture plane, show spherical cells but also mesenchymal-fusiform cells with invadosomes and no microvilli. The MDA-MB-231 cells seeded on the same substrate show only spherical cells but no microvesicles. Both the two cell groups penetrate into the collagen membrane within inter-fibre spaces. These data suggest that collagen array has a strong epigenetic effect on the phenotype of breast cancer cells.

ENDOTHELIAL GLYCOCALYX MODIFICATIONS AFFECT VASCULAR FUNCTIONS

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Endothelial dysfunction is one of the main triggers of atherosclerosis which is identified as a chronic inflammatory disease of the artery wall. However, the exact mechanisms underlying the impaired vascular structure and activity remain unresolved. Since inflammatory cytokine TNFα appears to be involved in the pathogenesis of atherosclerosis, we treated endothelial cells (HUVEC) with 0.1 μg/mL of TNFα for 24 hours to see its influence on extracellular matrix components as hyaluronan (HA) and syndecans (SDCs). TNFα in HUVEC leads to an increase of HAS2 expression and increase the HA in the pericellular coat suggesting that *in vivo* HA could be modified in the glycocalyx altering its adhesive properties. TNFα stimulation also affects syndecan4 (SDC4) core protein expression as well as its glycosaminoglycans (GAGs) chain composed mainly by heparan sulfate (HS). Moreover, the SDC4 overexpression after the pro-inflammatory stimulation correlates with a reduction in HUVEC permeability. As many functions of SDC4 depends on its GAGs chain, we found an upregulation of the critical enzymes involved in the synthesis of HS (*i.e.* EXT1, EXT2 and NDST1). Since NDST1 modifies GAGs chain, we measured by HPLC a significant increase of N-sulfation of HS residues. As N-sulfation levels in HS are involved in the inhibition of thrombus formation, we used HS extracted form HUVEC membrane and conditioned medium for platelets assays that revealed a decrease of platelets spreading. These data suggest that, during endothelial inflammation, the alteration of HA synthesis, SDC4 expression and its GAGs chains chemical composition can influence the platelets recruitments and, further, could promote the LDL accumulation during the onset of atherosclerosis. Since the hepatic enzyme PCSK9 is a novel pharmacological target for patients with cardiovascular disease lowering the plasmatic concentration of LDL, we examined the action of PCSK9 on HA metabolism and LDL receptors in HUVEC and smooth muscle cells (SMC).

LDLR and LOX1, the main LDL receptors, are influenced by PCSK9. Moreover, PCSK9 affected HA synthesis enzyme expression. These data indicate that PCSK9 could have a vaso-protective role regulating molecules involved in HA homeostasis.

THE LONG NON-CODING RNA HAS2-AS1 ACTS AS A COMPETING ENDOGENOUS RNA IN THE REGULATION OF BREAST CANCER CELLS AGGRESSIVENESS

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Hyaluronan (HA), a ubiquitous glycosaminoglycan of extracellular matrix, can modulate cell adhesion, motility, growth and inflammation. In tumour microenvironment, the up-regulation of HA synthases 2 (HAS2) and the overproduction of HA are often associated with tumour progression and metastasis. Recently, it has been discovered that the natural antisense transcript for HAS2 (HAS2-AS1) can modulate the expression of HAS2 and the production of HA in aortic smooth muscle cells via epigenetic modifications[1]. Although the role of HA and HAS2 in breast cancer is widely described, little is known about HAS2-AS1. We therefore studied the behaviour of MDA-MB-231 breast cancer cells upon the modulation of HAS2-AS1 expression by evaluating cell proliferation, migration and invasion, and analysed the expression of HA related genes and receptors. This analysis revealed that HAS2-AS1 knockdown stimulated the presence of a malignant phenotype, as its abrogation increased cell motility and invasion, as well as the expression of several HA related genes and the receptor CD44. These evidences suggested that HAS2-AS1 plays a role in breast cancer progression through alteration of HA metabolism. Moreover, long non-coding RNAs can orchestrate gene expression through a variety of mechanisms, regulating transcription and translation, chromatin remodelling and the interaction with other RNA species, i.e. miRNAs. HAS2-AS1 contains a putative binding site for miR-186, a negative regulator of the pro-apoptotic receptor P_2X_7[2]. The overexpression of HAS2-AS1 decreased the abundance of miR-186, suggesting a possible "sponge effect" exerted by HAS2-AS1 on mir-186 availability in the cytoplasm. In addition, the modulation of HAS2-AS1 stimulated the transcript levels of P_2X_7 along with other targets of mir-186, included some involved in cell cycle and apoptosis.
These data suggest that the "sponge effect" of HAS2-AS1 is able to antagonise the function of mir-186 on its downstream targets and could explain the presence of a malignant phenotype after HAS2-AS1 silencing in MDA-MB-231.

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HEPARAN SULFATE PROTEOGLYCANS AS TARGETS OF SELECTIVE PEPTIDES FOR CANCER DIAGNOSIS AND THERAPY

J. Brunetti[1], L. Depau[1], C. Falciani[1], G. Riolo[1], E. Mandarini[1], E. Karousou[2], A. Passi[2], A. Pini[1], L. Bracci[1]

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Cancer cell membranes and tumor associated extracellular matrix are characterized by a predominant presence of highly sulfated HSPG, which have already been identified as potential tumor markers in different cancers.
We previously reported on stable tetra-branched peptides named NT4, which bind with high selectivity to different human cancer cells and tissues and can efficiently and selectively deliver drugs or tracers for cancer cell imaging or therapy[1-4]. We found that the high selectivity of NT4 toward cancer tissues resides in their high affinity binding to sulfated glycosaminoglycans (GAG) with preferential binding to heparin and heparan sulfate[5,6]. Using drug-conjugated NT4 as selective tumor targeting agents, we obtained a significant reduction in tumor growth, compared with animals treated with the unconjugated drug under identical conditions[1,3,7]. Drug-conjugated NT4 can also by-pass drug resistance mediated by membrane transporters[8]. Besides being potential tumor markers, HSPG are also interesting potential drug targets since they appear to take part in many crucial events of cancer cell differentiation, such as epithelial mesenchymal transition, migration and invasion. We identified a putative sulfated oligosaccharide motif allowing high-affinity binding of NT4 to sulfated GAGs. NT4 was then used as a specific tool to analyze the role of sulfated GAGs in signaling events regulating oriented cancer cell migration. NT4 inhibits adhesion and migration of different cancer cells, dramatically affecting directionality and polarity of cell movement. We found that NT4 binding to sulfated GAGs abolishes directional migration, inducing disorganization of actin filaments and stress fibers and increasing the number filopodia. This happens without any change in activation of beta1 integrins or focal adhesion kinase, but with a decrease in Rac1 activity. Our results suggest that HSPGs work as primary conductors of directional cell migration by regulating Rac1 activity through an integrin-independent signaling pathway.

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PROTEOMIC CORRELATIONS OF GLYCOLYTIC ENZYME ISOFORMS WITH PROTEINS OF BREAST CANCER TISSUES

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The neoplastic cells undergo profound changes in metabolism, which from aerobic becomes predominantly anaerobic, according to the so-called "Warburg effect". To date, the most credited hypothesis to explain this phenomenon has been that of an adaptive response to the hypoxic environment of the tumor. However, in recent years the results of several studies have suggested that

the relative increase of the enzymes of the anaerobic metabolism could be one of the first steps occurring during the neoplastic transformation, rather than a secondary effect of the carcinogenetic process. Indeed, some of the glycolytic enzymes, namely G3P dehydrogenase, Enolase, Aldolase, may play relevant roles within some molecular and cellular mechanisms crucial for the development of the tumor. The aim of present work was to evaluate possible statistical correlations between the protein-expression levels of glycolytic enzymes with the other proteins identified (458) on the proteomic maps of 100 surgical fragments of breast cancer. Proteomic profiles were obtained by subjecting the protein extracts of the 100 BC surgical fragments to IPG-2D electrophoresis. The identification of the selected spots was determined by mass spectrometry. The statistical correlations were performed by applying the analysis of the Pearson coefficient. The results so far obtained showed that the expression levels of each proteomic isoform of the glycolytic enzymes identified is significantly correlated with proteins crucial for the breast cancer progression, involved in resistance to apoptosis, cell motility, responses to DNA damage, protein folding, and cell cycle control.
We believe that the results of the present study confirm and strengthen the evidence of the crucial role played by many enzymes of the glycolytic pathway in the breast cancer progression, and evidentiate for the first time the significance of protein isoforms in the intricate scenario of the breast cancer dynamics.

A PROTEOMIC APPROACH TO THE DETECTION OF DIFFERENTIALLY EXPRESSED PROTEINS IN TUMORAL AND NON-TUMORAL BREAST TISSUES

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The complex scenario of neoplastic progression makes necessary to search for new biomarkers useful for monitoring the clinical course of patients. The cancer research had uncovered many mechanisms of the tumor progression allowing the identification of entire protein classes functionally related to tumor development. The present study aimed to compare the proteomic profiles of breast cancer tissues with their adjacent non-tumoral counterparts, to identify possible alterations in the protein profiles. The surgical samples were obtained at the La Maddalena Hospital, according to the international recommendations. The proteomic procedures were performed using the IPG-2D electrophoresis followed by mass spectrometry for protein identification. The comparison between tumoral and non-tumoral proteomic profiles revealed a marked increase of over-expressed proteins in the tumor tissues, even though with a significant inter-individual heterogeneity. Interestingly, 60 proteins (out of the 458 identified) were more expressed in all the tumors versus the healthy counterparts; among these, were several glycolytic enzymes, some proteins involved in the resistance to apoptosis and others involved in response to stress. The association of glycolytic enzymes with the tumorigenesis was firstly reported by Warburg in 1935 and was attributed by the author himself, and others to follow, to the increase of the hypoxic conditions of the tumor environment. No other roles for these enzymes, outside the glycolysis, were at that time described. In the last years, it has been shown that many of them are engaged in different pathways crucial for the tumor progression.
The proteomic approach has proved to be a useful tool for the identification of isoforms of these enzymes, suggesting interesting speculations on the molecular mechanisms of the breast cancer progression.

MULTIMERIN-2 AS A GATEKEEPER OF VASCULAR STABILITY

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Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a hallmark of cancer. In the last decades efforts have been taken to halt this process to impair tumor growth. However, the modest survival benefits from anti-angiogenic therapy compels researches to develop new strategies. One of the most promising approaches aims at normalizing the abnormal and leaky vasculature associated with tumors to improve drug delivery and hence, therapy efficacy. In this context, Multimerin-2, whose expression is often lost in tumor-associated vessels, represents a key component. Multimerin-2 is an extra-cellular matrix protein deposited along blood vessels where exerts a homeostatic and angiostatic function. Our recent results indicate that Multimerin-2 is necessary to maintain endothelial cell-cell junctions stability. The depletion of Multimerin-2 in endothelial cells leads to dismantlement of the VE-cadherin lining causing a dramatic increase of cell permeability. The molecular mechanism elicited by the loss of Multimerin-2 involves the phosphorylation of VEGFR2 at Y951, recently connected to Src activation and the phosphorylation VE-Cadherin which, therefore is targeted for internalization and degradation. These results were corroborated *in vivo* since retinal vessels from *Multimerin-2*$^{-/-}$ mice displayed abnormal VE-cadherin lining and increased vascular leakage. Furthermore, B16F10 tumor-bearing *Multimerin-2*$^{-/-}$ mice showed altered vascular functionality, with an increased permeability and a reduced perfusion, compared to *wild-type* mice. The strikingly impaired vascular efficiency associated with a diminished chemotherapy efficacy in *Multimerin-2*$^{-/-}$ mice due to impaired drug delivery within the tumors. Taken together these results pinpoint Multimerin-2 as a key molecule for the maintenance of blood vessel stability and suggest the possibility to develop a marker of chemotherapy efficacy based on the expression levels of Multimerin-2 within the tumor vasculature.

ABROGATION OF EMILIN1- 4 1 INTEGRIN INTERACTION AFFECTS EXPERIMENTAL COLITIS AND COLON CARCINOGENESIS ENHANCING LYMPHATIC DYSREGULATION AND INCREASING INFLAMMATORY CASCADE

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Colon cancer is one of the principal types where a functional link between inflammation, tumor microenvironment and progression had been noted[1,2]; colitis-associated cancer is also related to striking changes in the lymphatic vasculature and dysfunction in the intestinal lymphatic network is a well-established feature of human inflammatory bowel disease[3,4]. The ECM protein EMILIN1 is expressed, among several other tissues, in the normal colonic mucosa; it controls elastogenesis, blood pressure homeostasis and is a key structural element in the maintenance of the integrity of lymphatic vessels[5,6,7]. It is an adhesive ligand of 4/9 1 integrins via its gC1q domain and this interaction down-regulates cell proliferation [8,9,10]. The impact of intestinal inflammation was analysed in *Emilin1*$^{-/-}$ (KO) and E933A EMILIN1 transgenic mice (E933A TG), in which a mutant EMILIN1, unable to be engaged by 4 1, is deposited. Mice were chronically treated with DSS 2% in the drinking water; KO and E933A-TG presented higher colitis scores and more severe mucosal injury, fibrosis and inflammatory infiltrates than WT. RNAseq analysis confirmed the up-regulation of several inflammatory response genes and the down-regulation of cell-cell adhesion molecules in E933A-TG. Colon tumours were induced with a single injection of AOM followed by 1-week exposures to 2% DSS. Tumour growth was observed over time by endoscopy and clinical scoring of colitis was performed applying the MEICS and DAI indexes. KO and E933A-TG mice had higher tumour incidence, bigger adenomas and less survival. Preliminary analyses on whole-mount colon specimens demonstrated that both KO and E933A-TG lymphatic vessels are irregular, dilated and characterized by dysmorphic structures and wide lacunae. This abnormal network architecture leads to enhanced lymphatic dysregulation, decreased lymph flow and impaired inflammatory cell drainage, providing a possible explanation for the riduced inflammatory resolution observed in KO and TG models.

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ALTERED HOMEOSTASIS IN OSTEOGENESIS IMPERFECTA OSTEOBLASTS FROM BRTL AND AMISH MURINE MODELS

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Osteogenesis imperfecta (OI) is a rare hereditary skeletal dysplasia, ranging from mild to perinatal lethal forms, and associated to dominant, recessive or X-linked transmission. OI is characterized by low bone mineral density, bone deformity, frequent fractures and short stature. Classical OI (types I to IV, based on Sillence classification) is a dominant disease caused by mutations in the *COL1A1* and *COL1A2* genes, encoding for α1 and α2 chains of type I collagen, the most abundant protein of the extracellular matrix (ECM). Mutated type I collagen is predominantly secreted in the ECM, leading to the impairment of its structural integrity, therefore it was traditionally considered the major cause of the OI bone phenotype. As observed in patients' fibroblasts and in murine and zebrafish models, mutations in collagen I genes are responsible for the delay in the molecule folding and its retention in the endoplasmic reticulum (ER) leading to increased level of post translational modifications[1, 2, 3]. The consequence of intracellular mutant collagen I retention is still poorly understood. Recently, we demonstrated that the OI patients fibroblasts homeostasis is significantly affected by cellular stress caused by mutant collagen retention and thus that cellular response to collagen retention is a key component in OI pathogenesis[1]. In this work, we dissected the cellular stress response to structurally altered collagen in primary osteoblasts (OBs) isolated from the Brtl and the Amish mice, two well established models for moderately severe OI type IV. They carry in heterozygosis a triple helical glycine substitution in two different collagen I chains: G349C in α1 chain in the Brtl mouse and G610C in α2 chain in the Amish model. Arrays of specific stress pathways such as Unfolded Protein Response (UPR), autophagy and apoptosis, validated by qPCR, western blot and immunofluorescence, revealed in both models the upregulation of several players of the analysed pathways. In order to alleviate cellular stress, the effect of sodium phenylbutyrate (4-PBA), a well known chemical chaperone, was also investigated in OBs. The treatment resulted in a partial rescue of the cell function, in particular decreasing the activation of the UPR stress sensor PERK and reducing apoptotic markers expression.
Our results demonstrate that the cellular response to collagen retention has a key role also in osteoblasts homeostasis.
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GENERATION AND CHARACTERIZATION OF OSTEOGENESIS IMPERFECTA TYPE XIV *IN VITRO* MODEL

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Osteogenesis imperfecta (OI) is a heritable bone disorder; the OI type XIV is a recessive form and is characterized by variable degrees of severity, multiple fractures and osteopenia[2]. OI type XIV is caused by mutations into *TMEM38B* gene encoding the endoplasmic reticulum (ER) trimeric cation channel TRIC-B. TRIC-B allows the transport of potassium ions across the ER membrane modulating the calcium flux. Defective ER calcium flux dysregulates collagen synthesis affecting the activity of multiple ER enzymes involved in its post-translational modification. Furthermore, calcium has been shown to be important for intracellular cell processes such us proliferation and differentiation[3]. Immortalized human Fetal Osteoblasts (hFOB 1.19 ATGC) were used to generate the *TMEM38B in vitro* knock-out (ko) model using the CRISPR/Cas9 system. Three RNA guides (gRNAs) were selected by *in silico* analysis and subcloned in the pSpCas9(BB)-2A-PURO (Addgene) vector that contains the Cas9 encoding sequence. The constructs were transfected in hFOB. Single cell clonal lines were obtained by serial dilution of the transfected cells and T7 endonuclease assay was used to identify positive clones. Proliferation of hFOB clones was performed by MTS assay. The intracellular calcium level and flux were analysed using FURA-2AM dye by fluorescence micro-

scope. L-[2,3,4,5-^{3}H]-proline was used for collagen metabolic labelling and SDS-PAGE for its analysis. qPCR and alkaline phosphatase (ALP) allowed the evaluation of different markers of osteoblastogenesis and the alizarin red staining was used to measure the mineralization. *TMEM38B* knock-out was generated and the lack of protein was confirmed by western-blot. The hFOB knock-out clones had a significantly reduced calcium concentration and they released significantly less calcium from the ER. The electrophoretic analysis of radiolabelled type I collagen from mutant clones showed a faster migration suggesting a lower level of post-translational modification. *TMEM38B* knock-out clones showed an impaired proliferation. The activity of ALP and the expression of *RUNX2*, *SP7*, *BLAGP* and *COL1A1* were reduced in ko clones. The alizarin red staining showed a reduced mineralization in mutant clones. We have successfully generated OI type XIV *in vitro* model using CRISPR/Cas9 system that will be useful to better understand the molecular basis of the disease.
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TRANSGLUTAMINASES IN MATRIX REMODELLING AND KIDNEY FIBROSIS

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The enzyme family of transglutaminases catalyse the post-translational modification of proteins, predominantly via cross-linking the γ-carboxamide group of a peptide-bound Gln residue to either the ε-amino group of a peptide-bound Lys residue on adjacent polypeptides, or a primary amino group of polyamine. Protein cross-linking depends on calcium binding and GTP dissociation, conditions that are favoured in the extracellular environment, or following cell injury and loss of calcium homeostasis. The most widespread member, transglutaminase-2 (TG2), has a well-established fibrogenic role in the kidney[1]. Increased synthesis and export of TG2 by tubular epithelial cells into the surrounding tubulo-interstitium is a significant feature of progressive kidney scarring[2]. Once outside the cell, TG2 accelerates the deposition of extracellular matrix (ECM) components, confers ECM-resistance to proteases and enhances transforming growth factor beta1 (TGF beta1) activation. As TG2 has a largely unknown unconventional export mechanism, we believe that this may be targeted to control its externalization in anti-TG2 therapy. To unravel the pathway leading to TG2 export we performed an unbiased analysis of the membrane interactome of TG2 in kidneys subjected to unilateral ureteric obstruction (UUO), an experimental model of kidney fibrosis. TG2-null and WT inbred C57BL/6J mice were subjected to UUO of the left kidney or a sham operation, and harvested at 21-day post-surgery. UUO kidneys were positive to alpha-smooth muscle actin and displayed a significantly higher level of active TGF-beta1. To identify TG2-associated proteins in the UUO model, we combined TG2-immunoprecipitation from whole kidney membrane preparations of WT and TG2-null kidneys (background controls) with quantitative proteomics by SWATH-MS. Differences between WT and TG2-null precipitated proteins were established by a paired sample z-test. A subnetwork of proteins responsible for endosomal transport were found to be associated with TG2 and formed significant clusters post UUO. Furthermore, the heparan sulphate proteoglycans syndecan-4 (Sdc-4) and perlecan were specifically associated with TG2[2,3]. The role of Sdc-4 in TG2 export was validated in NRK52 tubular epithelial cells upon siRNA-modulation[4]. These investigations led to the demonstration that TG2 is externalized via exosomes through a pathway dependent on Sdc-4.

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HYLURONAN: A SIMPLE MOLECULE WITH A COMPLEX REGULATION

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Changes in the microenvironment organization within vascular wall are critical events in the pathogenesis of vascular pathologies including atherosclerosis, restenosis and diabetic angiopathies. As the accumulation of hyaluronan (HA) into vessel wall supports neointima formation and cardiovascular diseases progression, we studied the intracellular regulation of HA synthesis mainly due to HA synthase 2 (HAS2). In previous studies, we showed that HA synthesis is strictly regulated by nutrients availability via HAS2 O-GlcNacylation and AMPK mediated phosphorylation[1]. Moreover, we found a critical role of HAS2-AS1 which opens to a further level of regulation via epigenetics epigenetics[2]. Recently, we found a complex multilevel regulation of HA synthesis we assayed whether HA production could be controlled by protein acetylation. Sirtuins play a key protective role in different pathologies as cardiovascular diseases[3]. These enzymes are NAD dependent deacetylases that target histones and non-nuclear proteins when the NAD+ levels increase. It is generally accepted that when nutrients are not limiting NAD is low whereas in case of nutrients shortage or caloric restriction NAD is high activating sirtuins. Since AMPK activity is closely connected to that of sirtuins[4], we decided to investigate whether HA synthesis could be regulated by sirtuins in human smooth muscle cells (SMC). As inflammation has a pivotal role in vascular pathologies driving recruitment of immune cells and SMC migration toward the neointima, we found that the chemical SIRT1 activator SRT1720 or resveratrol reduced monocytes adhesion and SMC migration after TNF-alpha treatments. As HA is critical to regulate immune cells adhesion and migration, we found that SRT1720 or resveratrol significantly reduced HAS2 as well as HAS2-AS1 expression and pericellular HA. Mechanistically, our preliminary data suggested that SIRT1 could alter NF-kB acetylation which remains in the cytoplasm inhibiting proinflammatory gene expression.

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THE IMMUNOMODULATORY ROLE OF TUMOR SYNDCAN-1 (CD138) ON *EX VIVO* TUMOR MICROENVIRONMENT CD4+ T CELL POLARIZATION IN INFLAMMATORY AND NON-INFLAMATORY BREAST CANCER PATIENTS

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The heparan sulfate proteoglycan Syndecan-1 (CD138) acts as a coreceptor for cytokines and chemokines modulating inflammation. Inflammatory breast carcioma (IBC) tissues displayed a higher expression of Syndecan-1 than non-IBC tissue and are characterized by a high infiltration of immune cells that may establish a chronic inflammatory tumor microenvironment mediating tumor progression and aggressiveness. In this study, we aimed to identify the immunoregulatory role of tumor Syndecan-1 in polarization of CD4+ T helper (Th) cell subsets isolated from the tumor microenvironment of IBC and non-IBC patients. Mononuclear cells drained from the axillary tributaries of non-IBC and IBC patients were isolated, and stimulated with the secretome of control and Syndecan-1-silenced SUM-149 cells (indirect co-culture). In addition, the axillary mononuclear cells of non-IBC and peripheral blood mononuclear cells (PBMCs) isolated from normal subjects were directly co-cultured with control and Syndecan-1-silenced Sum-149 cells. Using multicolor flow cytometry, we analyzed the expression of the intracellular IFN- , IL-4, IL-17, and Foxp3 markers as read out for Th_1, Th_2, Th_{17} and T_{reg} $CD4^+$ subsets, respectively.

Our flow cytometry results revealed that IBC displayed a lower basal Th_1 and Th_2 $CD4^+$ T cell subset frequency in blood drained from the tumor microenvironment in comparison to that from non-IBC patients. Syndecan-1-silenced SUM-149 cells significantly upregulated only the proportion of the T_{reg} ($Foxp3^+CD4^+$) subset of normal subjects in a direct co-culture relative to controls. However, relative to control SUM-149 cell, Syndecan-1 silencing significantly enhanced the polarization of Th_{17} ($CD4^+IL\text{-}17^+$), and T_{reg} ($Foxp3^+CD4^+$) subsets of non-IBC patients under both direct and indirect conditions. Moreover, it induced Th_1 (IFN- $^+CD4^+$) subset polarization under indirect conditions. $CD4^+$ T cells of IBC patients showed only an increased $Th_1/Foxp3^+T_{reg}$ ratio upon stimulation with the secretome of Syndecan-1-silenced SUM-149 cells relative to controls.

Overall, this study indicates a low frequency of the antitumor Th_1 subset in IBC and suggests that tumor Syndecan-1 has an immunoregulatory role in polarization of T helper cells within the tumor microenvironment that may have therapeutic implications in breast cancer.