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MMPs inhibition as molecular approach to abdominal aortic aneurysm (AAA) treatment

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Scientific background

Aortic aneurysm is a multifactorial degenerative disease characterized by destruction of aortic microstructure and subsequent wall dilatation. Because of the silent nature, the likely consequence of undiagnosed aneurysms includes rupture, which can be fatal and, thus, requires urgent surgical management. Aortic aneurysms are classified into two main groups depending on their location: thoracic aortic aneurysms (TAAs) and abdominal aortic aneurysms (AAAs). Aortic aneurysm account for 1% to 2% of all deaths in the Western countries and the incidence of thoracic aortic aneurysm (TAA) is ≈10.4 per 100 000 person years (Lindsay et al, 2011; Ramanath et al, 2009). AAAs have the highest incidence and are related with various risk factors, including hyperlipidemia, hypertension, sex and age. However, smoking is generally found as one of the most important risk factor (Blanchard et al, 2000; Lederle et al, 1997). Heritability estimates as high as 70% has been found, and recently, four genetic loci have been identified through genome-wide association studies (DAB2IP, LRP1, SORT1 and LDLR) (Gretarsdottir et al, 2010; Jones al, 2013; Bown et al, 2011; Bradley et al, 2013), and three candidate single-nucleotide polymorphisms have been identified as associated with AAA from meta-analysis (MMP9, ACE, MTHRF) (Thompson et al, 2008). In addition, a wide range of potential biomarkers for AAA have been identified, with two meta-analyses, repeating a significant association between AAA and several proteins (MMP2, MMP9, TIMP1, IL6,TNF-α, osteoprotegerin, osteopontin, IFN gamma, ICAM 1, VCAM1, D-dimer, CRP, α1-antitrypsin, fibrinogen, triglycerides) (Sidloff et al, 2014; Stather et al, 2014). Aneurysm is characterized by medial degeneration, determined by disaggregation of the lamellar organization of elastic fibers, infiltration of the adventitia and tunica media with inflammatory cells, degradation of the extracellular matrix (ECM), including both elastin fragmentation and disruptions of collagen, apoptosis of vascular smooth muscle cells (VSMC) and thinning of the tunica media (Bode-Jänisch et al, 2012). One of the reasons of AAA development is the deregulation of matrix metalloproteinases (MMPs) activity. MMPs belong to the family of proteolytic enzymes that degrade several components of ECM. The activities of MMPs are regulated at different levels: transcription, activation of the precursor zymogens and interaction with specific ECM components. Also, endogenous tissue inhibitors of MMPs (TIMPs) provide a balancing mechanism to prevent excessive ECM degradation. An imbalance between MMPs and TIMPs could lead to increase the MMPs activity and, thus, to pathological changes in the vessel wall structure associated with vascular disease (Raffetto and Khalil, 2008). The mammalian MMPs are a group of 23 structurally related enzymes that have a catalytic Zn^{2+} ion site (Nagase et al, 2006; Visse and Nagase, 2003). In principle, a combination of MMPs therefore has the ability to extensively degrade the ECM. All MMPs are synthetized as pre-pro-enzymes and secreted as inactive pro-MMPs. Many of the MMPs

have the ability to cleave and activate the pro-forms of other MMPs, thereby acting in protease cascades that could amplify their effectiveness (Nagase et al, 2006; Visse and Nagase, 2003). One of the features of MMP is that many of those genes are "inducibile" by a group of effectors such as growth factors, cytokines, chemical agents, physical stress, and oncogenic cellular transformation. Furthermore, pathological observations suggest that the earliest event may be the loss of elastin mediated by VSMCs, perhaps as a result of inflammatory activation, then followed by influx of leukocytes in response to the production of ECM fragments with chemotactic activities. This suggests a multi-stage (initiation and destabilization) paradigm for aneurysm growth and rupture. Initial loss of elastin in the medial layer causes compensatory fibrosis leading to normal or increased collagen deposition and consequently the loss the mechanical properties of the wall. Later, as a consequence of inflammation, destruction of all the major matrix components causes further distension and eventually rupture of the vessel wall. Consistently with this, imaging studies and pathological examinations of advanced AAAs demonstrate an association between leukocyte infiltration and likelihood of rupture (Hong et al, 2010; Reeps et al, 2008). Furthermore, different studies have revealed that also inflammatory processes have a pivotal role in the development of AAAs, which involves the infiltration of various immune cells (in particular macrophages and T cells) (Longo et al 2002; Manicone et al. 2008). Monocytes, circulating blood leukocytes that play an important role in the inflammatory response, have been implicated in the process of vascular remodeling and AAA expansion (Thompson et al. 1995; Golledge et al. 2008; Newby, 2008). Monocyte activity during inflammation is modulated by a variety of mediators such as cytokines, chemokines, and growth factors that allow interaction with the endothelium of the vessel wall through the upregulation of adhesion molecules. During this process, proinflammatory cytokines such as TNF-α and IL-1b promote the secretion of MMPs via the mitogen-activated protein kinase (MAPK) pathways (Chatzizisis et al. 2007). MMP2 is produced in the medial wall by SMCs, is activated by membrane-bound membrane type-1 (MT1-MMP) and inhibited by tissue inhibitor of metalloproteinases type 2 (TIMP-2). MMP2 has substrate specificity for elastin and fibrillar collagen (Aimes et al, 1995) and is found in the normal and aneurysm aorta in association with MT1-MMP and TIMP-2 (Crowther et al, 2000). However, MMP2 knockout mice are resistant to experimental induction of aortic aneurysms using a model of abluminal calcium chloride application. Reconstitution of these mice with wild-type bone marrow did not alter this resistance, suggesting that MMP2 derived from outside the bone marrow is a critical point (Jones et al, 2009). In aneurysm tissue there is an up-regulation of MMP2 production resulted from increased MMP2 transcription, with comparable levels of TIMP-2 and MT1-MMP mRNA. These data suggested that the regulation of MMP2 gene expression was altered in aortic SMCs from patients with abdominal

aortic aneurysm (Goodall et al, 2001). Moreover, MMP9 derived from inflammatory cells and MMP2 derived from vascular smooth muscle cells (SMCs) (Longo et al, 2002) have been shown to be critical factors required for the elastin destruction and proteolytic degradation, that are both hallmark features of AAAs, thereby leading to gradual aortic dilatation. Interestingly, such vascular wall degradation in human AAAs is often accompanied also by calcification of the aneurysmal wall, suggesting a possible link between aneurysm formation and calcification. Identification and treatment of AAA remain among the most prominent challenges in vascular medicine. Nowadays the pharmaceutical approach is not specific and includes the use of drugs acting to decrease the levels of blood pressure as esmolol, calcium channel blockers and nitroprusside (in emergency). Although the specific etiology of aneurysm formation is still unclear, the progression of aortic dilatation in the chronic phase probably results from a combination of hemodynamic stress, aortic injury, chronic inflammation, genetic propensity, and epidemiologic risk factors. One of the main methods to specifically inhibit MMPs is the use of RNA interference technique, where a small molecule of RNA (about 20-25 bp) inhibits gene expression by causing the destruction of mRNA molecule encoding the target protein. RNA interference (siRNA and shRNA) specifically suppresses gene expression in mammalian cells in vitro. These inhibitory effects result from selective degradation of target mRNA (McManus et al 2002). Recent studies show that shRNA can be used for the inhibition of gene expression in vivo. These results set the path for therapeutic applications, either locally or systematically (Dorn et al, 2004; Soutschek et al, 2004). MMP2s and MMP9s are interesting target for siRNA- or shRNA-based molecular therapy, since MMP2 is constitutively expressed in SMCs, and MMP9 is expressed by inflammatory cells. Efficient targeted delivery of therapeutic genes, by viral vectors, to specific cell types or tissues, is a major challenge for gene transfer in basic sciences as well as clinical gene therapy (Wiznerowicz, 2005). A major challenge of gene therapy concerns gene transfer systems able to efficiently trasduce cell and express transgene without harm to the recipient. Among the several viral vectors developed, lentiviral vectors (LVs) are attractive for gene therapy, because these are devoid of all wild-type lentiviral genes, infect non-dividing cells, integrate into the genome with high levels and long-term transgene expression, and accommodate relatively large genes (Follenzi et al, 2000). Moreover, selective targeting of specific cells such as hepatocytes by LVs limited immune responses against transgenes (Follenzi and Naldini, 2002). These vectors are effective tools for the delivery and sustained expression of transgene, both *in vitro* and *in vivo*, so far without detectable pathological consequences attributed to the vector. Significant progress has been achieved by eliminating non-essential viral sequences from the constructs, used to make vector, to improve safety, in optimizing the transfer construct, to improve transduction efficiency, in proving the therapeutic potential of

LVs mediated delivery in murine and human disease models (Biffi et al, 2013; Aiuti et al, 2013). *In situ* gene therapy of aneurysms involves selection of candidate genes, identification of target cells, and design of a means to transfer efficiently the desired gene into target cells. Vectors may be needed to transfer genes into cells, and promoter systems are required to regulate gene expression according to the therapeutic objectives. One specific challenge of vascular gene therapy is the difficulty involved in efficiently transferring the desired gene at the target site. A promising approach is the direct *in situ* transfer of genetic material into the vessel wall. Because of its relative simplicity, *in vivo* gene transfer is of interest for cardiovascular diseases. In fact, no cell harvesting or culture expansion is required. We hypothesized that altering expression of MMP would affect extracellular matrix remodeling, allowing us to control AAA disease progression and thereby potentially offer a novel molecular therapy.

Aim of the project

This study aimed to detect the involvement of MMPs in human AAAs. We evaluated the concept of LV directed gene transfer of shRNA to MMP2 and MMP9 for aneurysm molecular therapy. LVs carrying MMP2-shRNA and MMP9-shRNA *in vitro* have been used.

METHODS

Samples collection:

Human samples of AAA were surgically recovered; control aortic tissues were obtained from autopsy. All samples were kindly provided by the Vascular Surgery Unit, S. Orsola—Malpighi University Hospital (Bologna), after obtaining Local Ethic Committee Approval (APP-13-01)

Immuno-histological assay

Tissue samples were rinsed in phosphate-buffered saline (PBS) and fixed in 4% formalin for 48h at 4° C. The samples were subjected to several dehydration steps of 60 min incubation in solutions with increasing ethanol content (50%, 70%, 95% and 100%) and 1h incubation in xylene. The specimens were embedded in paraffin (Sherwood Medical, St. Louis, MO, USA) and serial sections were cut with a microtome (Leica- Jung, Germany) in consecutive 5 mm-thick sections. Rehydrated sections were stained with hematoxylin and eosin, Sirius Red, Masson's trichrome and AB pas staining. Weigert and AB pas staining were performed in the laboratory of Prof. Boldorini. For Sirius red, sections were then examined using a polarizing microscope. In addition, immunofluorescence and immunohistochemistry were performed on deparaffinized sections. For immunofluorescence, samples were processed with primary anti-elastin (Diagnostic Biosystems, CA, USA), MMP2 and MMP9 (Millipore, Italy) antibodies followed by a FITC- or TRITC-conjugated secondary antibody (Vector, CA, USA) incubation. DAPI (4',6-Diamidino-2-phenylindole) was used for nuclear staining. For immunohistochemistry, samples were processed with primary MMP9 antibody, followed by a secondary antibody (Vector, CA, USA) incubation. The reaction was detected using the avidin–biotin method. All images were acquired using Pannoramic MIDI 3DHISTECH.

Gelatin zymography for detection of MMP2 and MMP9

Non-reducted conditioned media samples were resolved by SDS-PAGE gels containing gelatin. After electrophoresis, gels were incubated with TRITON X-100 for 3h at room temperature, and then incubated in a solution of $CaCl_2$ (1mM) and NaCl (15mM), pH 7.4 overnight at 37°C. After incubation, gels were fixed and then stained with Coomassie stain. For objective quantification ImageJ software was used.

Lentiviral vector transduction

Lentiviral vectors (LV) were designed in the laboratory of Prof. Follenzi. $5*10^4$ C2C12 (myoblast) and RAW 246.7 (monocyte) were transduced with LV. We tested the transduction efficiency using different dilution of LV for the expression of green fluorescent protein (GFP) under the control of

the phosphoglycerate kinase (PGK) promoter. After 3 days, cells were detached and resuspended in 1% FBS and 0,1% of sodium azide, then analyzed with a FACScalibur flow cytometer (BD Biosciences, Italy).

Then C2C12 and RAW 264.7 were transduced with LV-sh-MMP2 and LV-sh-MMP9 respectively. Five different clones were used (all from Mission, Sigma). After transduction process, cells were selected with puromycin (3μg/mL for C2C12 and 2.5μg/mL for RAW 264.7 cell line).

After cell selection, the levels of MMP2 were evaluated in differentiated C2C12 cells. For differentiation experiment, C2C12 were cultivated in DMEM + 10% FBS (growth medium, GM) until an 80–90% confluence was reached, then were shifted to DMEM + 2% horse serum (differentiation medium , DM). After differentiation, cells were set in serum-free medium for 24h.

The levels of MMP9 were evaluated with RAW 264.7 cells. Briefly, $6*10^4$ cells/cm^2 were seeded and after 24h were stimulated with lipopolysaccharide (LPS 100ng/mL) in serum-free medium for 24 h.

MMP2 level

The concentration of pro-MMP2 and MMP2 was measured in conditioned medium without FBS by ELISA immunoassay according to manufacturer instructions (Abcam AB100730, Italy).

Western blot

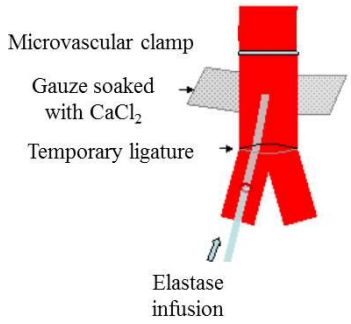
Cells were lysed in hot SDS buffer (1% w/v SDS, 100 mM Tris–HCl, pH 7.0). Protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). 50 μg total proteins in sample buffer (62.5 mM Tris–HCl, pH 6.8, 20% glycerol, 5% β-mercaptoethanol, 0.5% bromophenol blue) were separated in SDS–PAGE electrophoresis and transferred to a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). Blotted proteins were blocked with 5% non-fat dried milk in PBS, pH 7.4, for 1h at room temperature and incubated overnight with primary antibody. After washing three times with PBS 0.1% Tween 20, the membranes were incubated with secondary antibody–peroxidase conjugates for 1h at room temperature. Protein bands were visualized using ECL detection reagents in a chemisensitive visualizer (VersaDoc, BioRad, Italy).

Mouse Models of abdominal aortic aneurysm

AAA was induced in C57BL/6 mice. Mice were divided in two different groups based on the treatment. The infra-renal aorta above the iliac bifurcation of mice was treated with: $CaCl_2$ and elastase (aneurysm group) and PBS (sham group).

Mice were anesthetized using intraperitoneal injection of Xilazine (15 mg/kg), Ketamin (100 mg/kg) e Buprenorphine (0.05 mg/kg) and then placed under an operating microscope (Leica). A long midline abdominal incision from pubis to xiphoid was made, and the abdominal cavity was exposed. The intestines were retracted superiorly and covered with wet gauze to keep them moist during the surgery. The abdominal aorta was exposed and gauze was placed on the aorta, soaked with a solution of $CaCl_2$ (0.5M) or PBS, for 15 minutes (Wang et al, 2013). For intraluminal elastase infusion, the isolated region of the aorta was filled with 30 μL of porcine pancreatic elastase (135 U/mg; Elastin Products, Mo) through catheter without any aortic expansion. After 20 minutes of intraluminal static elastase infusion and extraluminal $CaCl_2$ exposure, the catheter was removed and the right femoral artery was ligated. The microvascular clamp and the temporary ligature were removed from the aorta to restore blood flow.

The intestines were then returned to the abdominal cavity. The abdominal incision was closed with continuous running 5-0 nylon suture and the mice were placed in warmed cages to recover. At 4 weeks after the operation, aortic tissues were excised for histological examination.



Schematic representation of surgery in mouse models

Results and Discussion

Immuno-histological assay

The vessel wall structural modifications in presence of abdominal aneurysm with respect to undamaged aortas have been evaluated. Figure 1 shows that the presence of aneurysm completely modified the vessel wall microstructure. As shown by haematoxylin-eosin staining, the aortic aneurysm (B and C) is characterized by a marked inflammatory response, as suggested by the presence of numerous inflammatory cells identified as leukocyte. There is a loss of smooth muscle cell (elongated nuclei) in aneurysm samples while, in the control, SMCs are aligned in the elastic lamina (A). Weigert staining is used for elastic fiber detection. The elastic fibers, in the media layer, are observed to be disrupted and fragmented with irregular arrangement (F). In control aorta, elastic fibers are visualized as black lines (D). The analysis with the Masson trichrome shows thickening of

collagen fibers in the medial layer of aneurysm samples (I) with respect to control (G). Alcian-blue pas staining has shown an accumulation of acidic mucopolysaccharides, indicating cystic medial degeneration, in particular in the atheroma (K), present under the endothelial layer.

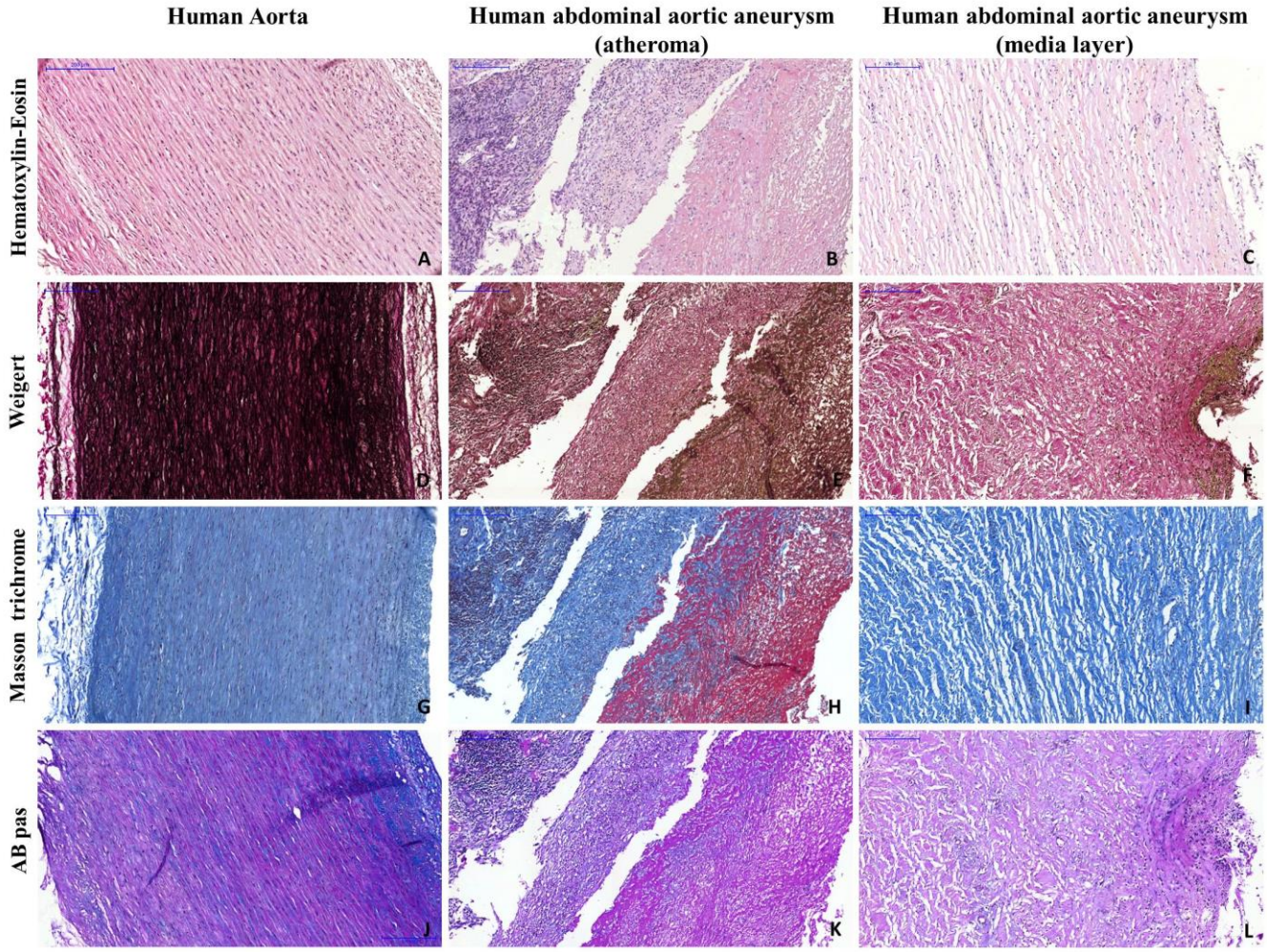


Figure 1. Representative images of Hematoxylin-eosin staining (A-B-C), Weigert for elastic fiber detection (D-E-F), Masson Trichrome (G-H-I), AB pas (J-K-L) staining.

Picro-Sirius red staining (Figure 2), observed with polarized light, confirmed a degradation of smaller collagen type III fibers (green fibers) in aneurysm samples, and an accumulation of collagen type I (red fibers).

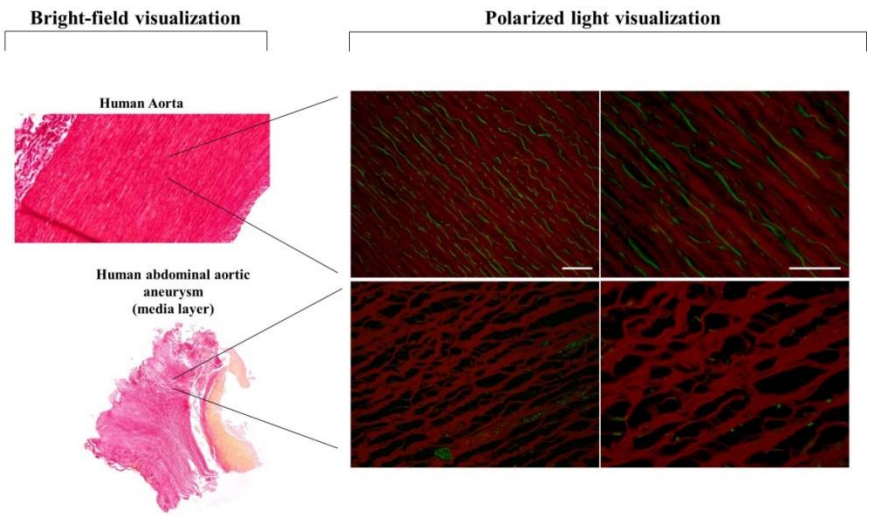


Figure 2. Picro-Sirius Red staining for collagen visualization.

We have analyzed MMP2, MMP9 and elastin expression using immunofluorescence assay on paraffin-embedded samples (Figure 3). As already shown in Weigert staining, elastin results disrupted and MMP9 is over-expressed in human aneurysm with respect to control. No significant differences are observed in MMP2 expression in both aneurysm and control samples.

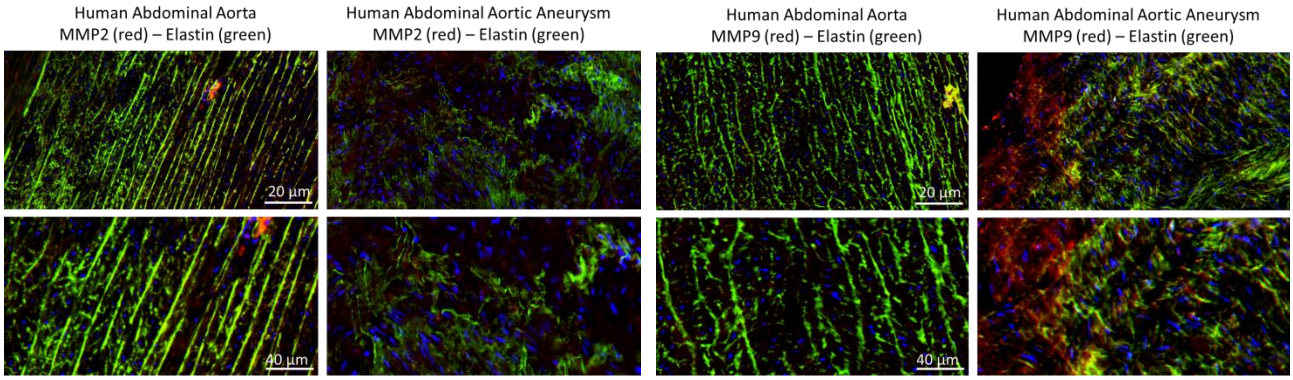


Figure 3. Representative images of immunofluorescence analysis.

Further analyses for MMP9 expression were performed. In figure 4 (A and B), zymography and immunohistochemistry on human aorta and abdominal aortic aneurysms are shown. These results confirm the key role of MMP9 in aneurysm progression.

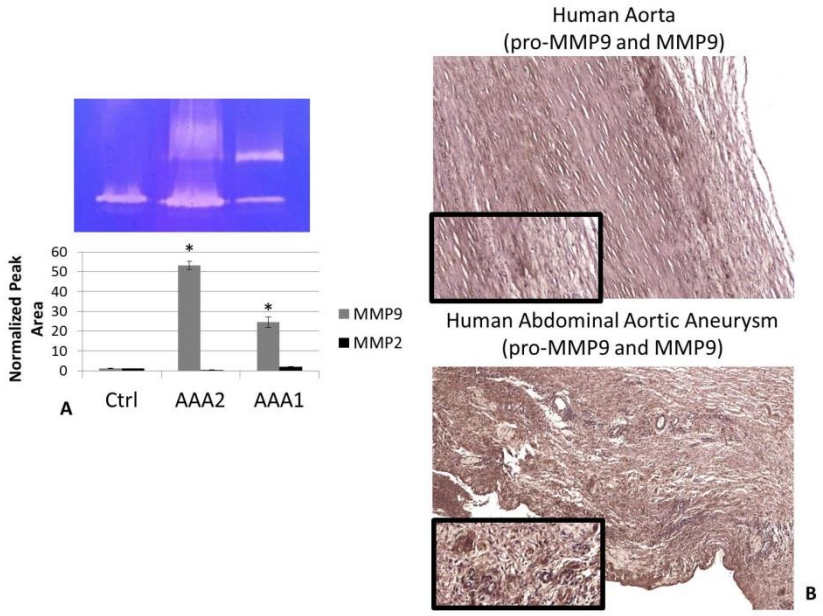


Figure 4. Zymography (A) and relative densitometry, representative images of immunohistochemistry assay (B) on paraffin embedded tissue

LV Transduction in C2C12 cell line

For cytometric analyses, a third generation lentiviral vectors (LV) system, expressing GFP under the control of PGK promoter, have been designed. Results obtained with C2C12 cell line, using serial dilution of LV, show a 92,27% of GFP positive cells indicating a very high transduction efficiency, using one-half diluted LV-PGK-GFP.

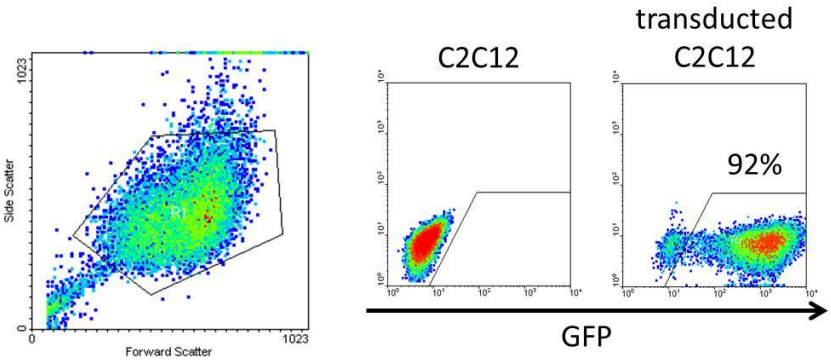


Figure 5. **Facs analyses on transducted C2C12 with LV-PGK-GFP**

MMP2's expression was induced in differentiated myotubes. MMPs expression on C2C12 cells, treated with five different LV-shRNA-MMP2, show a deregulation of MMP2 translation with respect to control. As shown by zymography assay, the highest silencing efficiency is obtained by LV-shRNA3 and LV-shRNA4. However, the levels of MMP2 were not as low as expected.

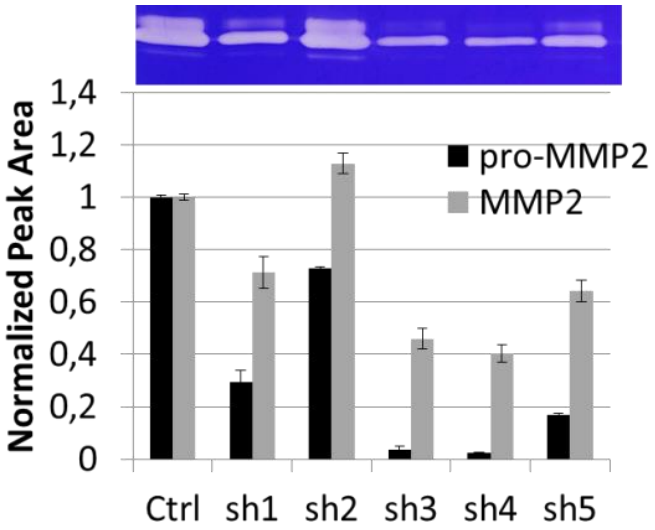


Figure 6. Zymography assay on transducted C2C12 with different five LV-shRNA.

Co-transduction

C2C12, previously transduced with LV-shRNA3 and LV-shRNA4, were co-transducted with the same LV-shRNA in different combinations: shRNA3+shRNA3 (sh3+sh3), shRNA3+shRNA4 (sh3+sh4), shRNA4+shRNA3 (sh4+sh3), shRNA4+shRNA4 (sh4+sh4). Using this co-transducted cell lines, several tests have been performed: zymography (A); E.L.I.S.A. (B) performed on supernatant media and western blot (C) on total cell lysate shown that sh3+sh3 and sh3+sh4 are the best combinations for the silencing of mouse MMP2 proteins.

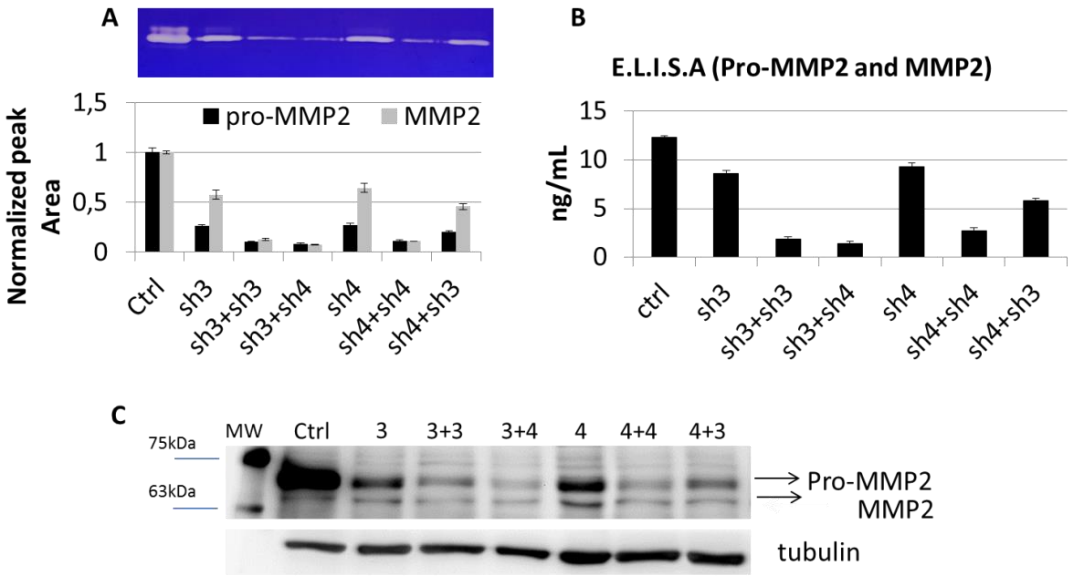


Figure 7. Validation of sh-RNA in RAW cell line

LV Trasduction in RAW cell line

Results obtained with RAW cell line show an 87% GFP of positive cells, indicating a very high transduction efficiency, using one-half diluted LV-PGK-GFP. For MMP9 inhibition experiments, MMP9's expression was induced in RAW stimulated with LPS (100ng/mL). MMPs expression on RAW cells, treated with five different LV-shRNA-MMP9, show a significant inhibition of MMP9 transduction with respect to control. As shown in zymography and western blot assay, the best silencing effect is obtained by the use of LV-shRNA1.

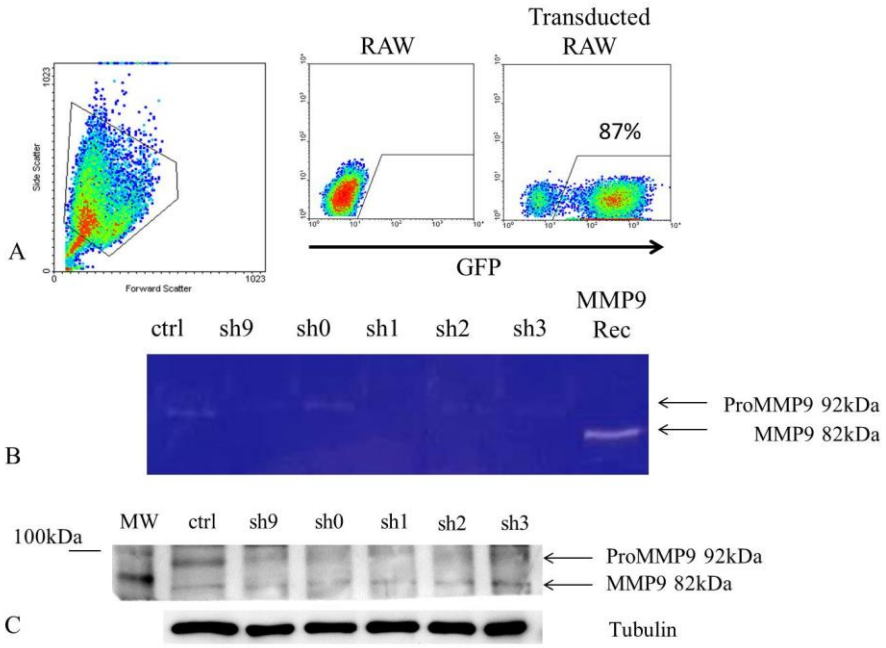


Figure 8. LV-shRNA validation in RAW cell line.

Mouse model of AAA

In mouse model of AAA, the structural remodeling in the aorta was investigated by assessing the integrity of the aortic wall by immunofluorescence assay. Compared with the sham group, $CaCl_2$ and elastin treated mice resulted in excess dilatation of the aortic lumen, loss of elastin lamella structure and presence of MMP2 and MMP9 in the whole vessel wall structure.

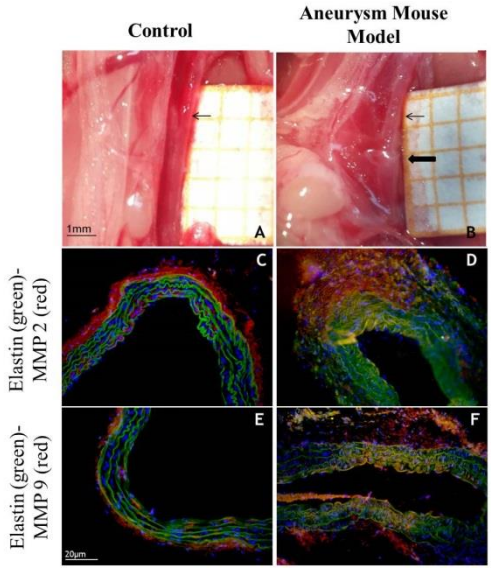


Figure 9. Mouse model of abdomianal aortic aneuryms

Conclusions:

Our data obtained on human samples confirmed the key role of MMP9 in aneurysm developing. MMP-9 levels significantly correlate with AAA. However, the utility of MMP-9 as a diagnostic test is limited due to low sensitivity and specificity. LV transduction is a very efficient method to obtain gene silencing and the use of this technique represents an innovative approach in the treatment of aneurysms by molecular therapy.

Thoracic and abdominal aortas have different characteristics that alter their susceptibility to different pathological stimuli. This aspect is associated with a differential ECM turnover rate in thoracic versus abdominal aorta. Notably, genetic disorders that interfere with collagen or elastin production or assembly are often associated with TAA and less frequently with AAA. This differential susceptibility of abdominal aorta to excess proteolysis could also suggest potential therapeutic advantages for reducing MMP activities in patients with AAA by MMP inhibitors.

Future perspectives:

Future studies will involve the interactions between lymphocytes (isolated from peripheral blood samples) and Vascular Wall Mesenchymal Stromal Cells isolated from AAA wall (AAA-MSCs) of patients who undergo surgical repair. Immunomodulation, MMP9 expression and its own silencing will be performed in AAA-MSCs after co-culture with activated peripheral blood mononuclear cells. Moreover, the LV behavior in *ex-vivo* three-dimensional environment will be investigated.

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- Decellularized biological matrices: an interesting approach for cardiovascular tissues repair and regeneration.
  Boccafoschi F, Botta M, Fusaro L, Copes F, **Ramella M**, Cannas M, J Tissue Eng Regen Med 2015; in press

- An optimized table-top small-angle X-ray scattering set-up for the nanoscale structural analysis of soft matter.
  Sibillano T, De Caro L, Altamura D, Siliqi D, **Ramella M**, Boccafoschi F, Ciasca G, Campi G, Tirinato L, Di Fabrizio E, Giannini C. Sci Rep. 2014 Nov 10;4:6985. doi: 10.1038/srep06985. PMID: 25382272

- Arginine-glycine-glutamine and serine-isoleucine-lysine-valine-alanine-valine modified poly(l-lactide) films: bioactive molecules used for surface grafting to guide cellular contractile phenotype. Boccafoschi F, Fusaro L, Botta M, **Ramella M**, Chevallier P, Mantovani D, Cannas M. Biointerphases. 2014 Jun;9(2):029002. doi: 10.1116/1.4864432. PMID: 24985206

- Human elastin polypeptides improve the biomechanical properties of three-dimensional matrices through the regulation of elastogenesis. Boccafoschi F, **Ramella M**, Sibillano T, De Caro L, Giannini C, Comparelli R, Bandiera A, Cannas M. J Biomed Mater Res A. 2014 Jun 10. doi: 10.1002/jbm.a.35257. [Epub ahead of print] PMID: 24913186

- Short-term effects of microstructured surfaces: role in cell differentiation toward a contractile phenotype. Boccafoschi F, Rasponi M, **Ramella M**, Ferreira AM, Vesentini S, Cannas M. J Appl Biomater Funct Mater. 2014 Apr 18:0. doi: 10.5301/JABFM.5000186. [Epub ahead of print] PMID: 24756781

- Biological evaluation of materials for cardiovascular application: the role of the short-term inflammatory response in endothelial regeneration. Boccafoschi F, Mosca C, **Ramella M**, Carmagnola I, Chiono V, Ciardelli G, Cannas M. J Biomed Mater Res A. 2013 Nov;101(11):3131-40. doi: 10.1002/jbm.a.34630. Epub 2013 Mar 25. PMID: 23529998

- The effect of mechanical strain on soft (cardiovascular) and hard (bone) tissues: common pathways for different biological outcomes. Boccafoschi F, Mosca C, **Ramella M**, Valente G, Cannas M. Cell Adh Migr. 2013 Mar-Apr;7(2):165-73. doi: 10.4161/cam.23020. Epub 2013 Jan 3. Review. PMID: 23287581

CONGRESS:

BACCAM A, **RAMELLA M**, BOCCAFOSCHI F, MERICSKAY M, LI Z, COLETTI D. Molecular pathways involved in the crosstalk between cytokines and mechanical cues in cancer cachexia. In 8th Cachexia Conference, Paris 4-6 December 2015.

BOCCAFOSCHI F, BOTTA M, FUSARO L, **RAMELLA M**, TORRI F, COPES F, AZZIMONTI B, CANNAS M (2015). Decellularized matrices for tissue engineering enriched with antibiotics: a promising approach for tissue regeneration. In SIB, Ancona, 3-5 June 2015.

BOCCAFOSCHI F, **RAMELLA M**, FUSARO F, BOTTA M, GIANNINI C, BANDIERA A, CANNAS M (2014) Elastin-like polypeptides improve the mechanical properties of three-dimensional matrices through the regulation of elastogenesis. In: Termis Congress 2014 Tissue Engineering and regenerative medicine, Genova (Italy), 10-13 June 2014.

BOCCAFOSCHI F**,** BOTTA M, FUSARO L, **RAMELLA M** (2014). Effects of Des-Acyl Ghrelin on vascular cells. In 41st Annual ESAO Congress, Roma, 17-20 September 2014.

LESSONS

- "Tissue engineering: the state of the art" – 14 November 2014 – Dott.ssa Francesca Boccafoschi - Department of Health Sciences, University of Eastern Piedmont.
- "Regenerative Medicine" – 21 November 2014 – Prof. Maria Prat - Department of Health Sciences, University of Eastern Piedmont.
- "Ribosomopathies" – 25th May 2015 – Prof. Steve Ellis – Medical School, University of Louisville (Kentucky)
- "Basis of scientific research" – 10th June 2015 – Prof. Nicoletta Filigheddu – Università del Piemonte Orientale (Italy)

SEMINARS

1. "Dysregulated antigen receptor signaling: molecular lessons from two congenital lymphoproliferative disorders" – 06 November 2014 - Prof. Andrew L. Snow - Department of Pharmacology Uniformed Services University of the Health Sciences Bethesda (Maryland, USA).
2. "Optical coherence tomography from bench to bedside shening the light during percutaneous vascular intervention" – 17 November 2014 - Dott. Secco Gioel Gabrio – Department of Health Sciences, University of Eastern Piedmont.
3. "Nuove sfide ed opportunità dell'epidemiologia molecolare per lo studio dei tumori" – 27 November 2014 - Prof. Laura Baglietto - Inserm - Centre for Research in Epidemiology and Population Health, Unit: Nutrition, Hormones and Women's Health, Paris.
4. "Humoral responses to HCV infection and clinical outcomes" – 28 November 2014 - Dott. Arvind Patel - Programme Leader, MRC Centre for Virus Research, University of Glasgow (UK).

5. "Uncovering the role of β-HPV in field cancerization: a collaboration in progress" – 4 December 2014 – Dott. Girish Patel - European Cancer Stem Cell Research Institute, Cardiff (UK).
6. "Focus on the liver: from basics of NAFLD to hot topics in HBV & HCV infections" – 5 December 2014 – Prof. Rifaat Safadi M.D, Chairman of the Israeli Association for the Study of Liver. Director of Liver Unit. Institute of Gastroenterology and Liver Diseases. Division of Medicine. Hadassah Medical Organization, Hadassah Hebrew University Medical Center, Jerusalem.
7. "From the legend of Prometheus to regenerative medicine" – 16 December 2014 – Prof. Antonio Musarò, DAHFMO-Unit of Histology and Medical Embryology Sapienza University of Rome
8. "Proof of principle for cell therapy: from autologous transplantation of tissue specific progenitors to gene corrected patient specific injured pluripotent stem cells" – 11 March 2015 – Prof. Darko Bosnakovski - Associate Professor, University "Goce Delcev" Stip, Faculty of Medical Sciences, Krste Misirkov bb, 2000 Stip R. Macedonia.
9. "Signal control in iNKT cell development and function" – 09 April 2015 - Prof. Xiaoping Zhong, MD, PhD - Associate Professor, Department of Pediatrics-Allergy and Immunology Duke University, Medical Center, Durham (North Carolina, USA).
10. "Actin-based mechanisms in the control of gene expression and cell fate" – 21st April 2015 – Prof. Piergiorgio Percipalle – Associate Professor, Department of Cell and Molecular Biology, Karolinska Institutet (Solns, Sweden).
11. "An integrated approach to the diagnosis and treatment of ovarian cancer" – 7th May 2015 – Prof. John McDonald, MD, PhD – Integrated Cancer Research Center, School of Biology and Parker H. Petit Institute of Bioengineering and Biosciience, Georgia Institute of Technology, Georgia Tech University, Georgia (Atlanta, USA).
12. "Recent developments in (cutaneous) Human Polyomavirus research" – 5th June 2015 – Mariet C.W. Feltkamp – Associate Professor of Medical Virology, Department of Medical Microbiology, Leiden University Medical Center (Leiden, The Netherlands).
13. Miniworkshop on "Biotechnology for Dermatology" – 9th July 2015 - Dr Gwenaël ROLIN, PhD - Clinical Research Engineer - Thomas LIHOREAU - Ingénieur hospitalier, Research and Studies Center on the Integument (CERT), Department of Dermatology, Clinical Investigation Center (CIC INSERM 1431), Besançon University Hospital; INSERM UMR1098, FED4234 IBCT, University of FrancheComté, Besançon, France.
14. "High-tech product preservation and operator protection: two apparently opposite requirements in different fields of medicine and biotechnology: the emerging glove box approach" – 15th July 2015 - Dr. Ing. Marco Fatta, Phd – COMECER Group (Italy).
15. "Le cellule staminali nel danno renale acuto e nel trapianto di rene" – 28th July 2015 - Dr. Vincenzo Cantaluppi, MD – Facoltà di Medicina e Chirurgia, Università di Torino (Italy).
16. "Cell based models for studying molecular mechanisms of Facioscapulohumeral Muscolar Distrophy (FSHD)" , "Toward animal model for Facioscapulohumeral Muscolar Distrophy (FSHD)" – 3rd September 2015 - Prof. Darko Boshnakovski, PhD – University Goce Delcev Stip, Faculty of Medical Sciences (Stip, R. Macedonia).