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Endothelial MMP-9 Drives the Inflammatory Response in Abdominal Aortic Aneurysm (AAA)

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Abstract

Abdominal aortic aneurysm (AAA) is a complex multi-factorial disease leading to life- threatening complications. Chronic inflammation and extracellular matrix degradation are the major pathological features of AAA. Vascular inflammation involves complex interaction among inflammatory cells (i.e. neutrophils, lymphocytes, monocytes, macrophages), endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and extracellular matrix (ECM). Although vascular endothelium plays a key role in aneurysm disease, the molecular mechanisms underlying its involvement is only partially understood. In this study, we have characterized the role of matrix metalloproteinase-9 (MMP-9) as potential trigger of the inflammatory response during the reciprocal interaction between ECs and VSMCs. Briefly, in biopsies of human AAA we found increased level of MMP-9, IL-6 and monocyte chemoattractant protein-1 (MCP-1), which correlated with a massive medial neo-angiogenesis. In particular, *in vitro* silencing of MMP-9 in ECs, using specific shRNA delivered by lentiviral vectors, inhibited TNF-alpha mediated activation of NF-kB. In addition, ECs void of MMP-9 failed to migrate in 3D matrix and affected VSMC behavior in terms of matrix remodeling. Overall our findings indicate that silencing of MMP-9 may represent a therapeutic target to restore vascular extracellular matrix remodeling.

Riassunto

L'aneurisma addominale aortico (AAA) è una malattia multi-fattoriale complessa che porta a complicanze rischiose per la vita. Le principali caratteristiche patologiche dell'AAA sono l'infiammazione cronica e la degradazione della matrice extracellulare.

L'infiammazione coinvolge una complessa interazione tra cellule infiammatorie (in particolare neutrofili, linfociti, monociti e macrofagi), cellule endoteliali (ECs), cellule vascolari muscolari lisce (VSMC) e la matrice extracellulare (ECM). Anche se l'endotelio vascolare svolge un ruolo chiave nello sviluppo dell'aneurisma, i meccanismi molecolari alla base del suo coinvolgimento sono solo parzialmente delineati. In questo studio abbiamo caratterizzato il ruolo delle metalloproteinasi-9 (MMP-9) endoteliale come potenziale agente promuovente della risposta infiammatoria durante l'interazione tra EC e VSMC. In particolare, abbiamo rilevato in biopsie di AAA una maggiore espressione di MMP-9, IL-6 e *monocyte chemoattractant protein-1* (MCP-1), che correla con una massiccia neo-angiogenesi presente anche nello strato medio della parete vascolare. È stato valutato il silenziamento *in vitro* di MMP-9 in EC, utilizzando specifici shRNA veicolati da vettori lentivirali, ed è stata osservata un'inibizione nell'attivazione di NF-kB a seguito di stimolazione con TNF-alfa.

Inoltre, le cellule endoteliali private di MMP-9 non sono in grado di migrare in una matrice 3D, e influenzano il comportamento VSMC in termini di rimodellamento della matrice. Nel complesso i nostri risultati indicano che il silenziamento dell'MMP-9 endoteliale potrebbe rappresentare un bersaglio terapeutico per ripristinare il rimodellamento fisiologico vascolare della matrice extracellulare.

1. Introduction

1.1 Macro- and micro-anatomy of human aorta

1.1.1 Human aorta

The aorta is the largest artery of the human vascular system and it is designated to deliver blood from heart to the systemic circulation. The human aorta arises from the left ventricle and descends to the abdomen; it can be distinguished into distinct segments: ascending aorta (between the left ventricle and the aortic arch), aortic arch, descending thoracic aorta (above the diaphragm) and abdominal aorta (below the diaphragm). The aorta belongs to the elastic arteries, which are large-diameters vessels (larger than 2.5 cm) with close localization to the heart. Healthy aorta of an adult normally measured roughly 3 cm diameter at the origin (ascending aorta), 2.5 cm along the descending thoracic aorta and 1.8-2 cm along the abdominal aortic segment. Elastic artery structure is kept by a network of connective fibres, elastin and collagen, that contributes to the arterial distension and recoil during left ventricle contraction (systole) and relaxation (diastole) respectively; moreover, the elastic artery recoil allows the blood entering into the small arteries. Conversely, the muscular arteries are located at periphery and provide blood to the whole tissue (skeletal muscle and inner organs). Muscular arteries are medium-size vessels (about 0.4 cm) and they are mainly composed of smooth muscle. This structure is functional to the muscular artery ability to vary the laminal diameter depending blood pressure, and regulate blood flow to each organ.

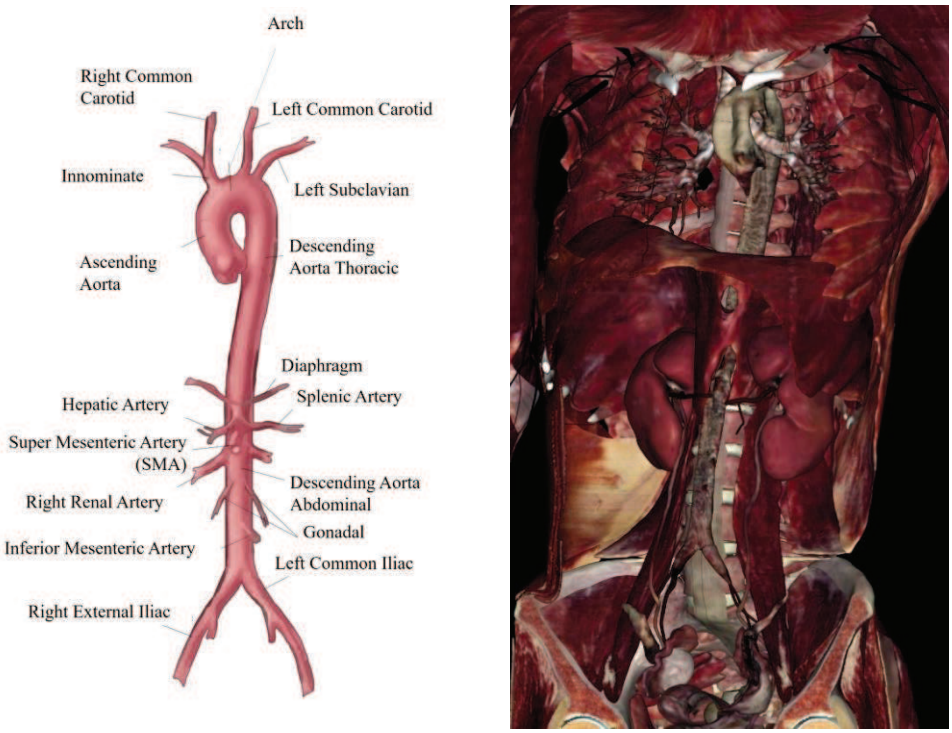


Figure 1. Human aorta anatomy. Diaphragm divides aorta in two portions: thoracic and abdominal aorta. Virtual dissection technology from Anatomage Table (Anatomage, CA, USA)

1.1.2 Structural features of human arterial wall

The healthy wall of aorta is composed by three layers called tunicae: intima, media and adventitia (Gasser et al., 2006)

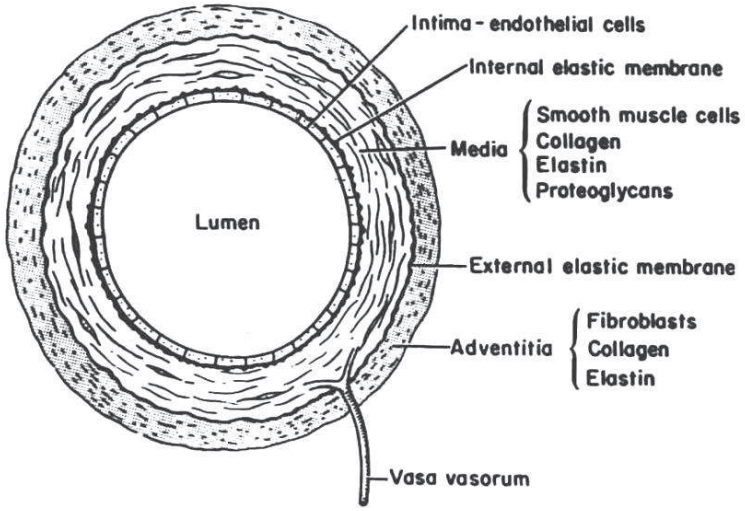


Figure 2. Microanatomy of a vessel wall.

Three layers compose arterial wall. The intima and the media are separated by the internal elastic membrane. The external elastic membrane separates the media and the adventitia.

(https://iame.com/online-courses/ultrasound-vascular/vascular-laboratory-markers-of-cardiovascular-risk)

Tunica intima is the innermost layer of the artery wall; it consists of a single layer of endothelial cells (ECs) placed on a thin basal lamina and a sub-endothelial layer of various thickness depending on localization, age and pathological changes. ECs compose the luminal side of the vessel wall and they are in contact with the blood flow; ECs are characterized by an elongated flat shape, that can undergo changes depending on the direction of the blood flow. ECs communicate to each other through a network of three types of junctions: I) *tight junctions*, or zonulae occludens, regulating the substances delivery across the endothelium; II) *adherens junctions*, whose main component is given by cadherins; they are designated to control the endothelium permeability to circulating cells; III) *gap junctions*, structures designated to allow ions and metabolites exchange between cells (Bazzoni and Dejana, 2004). ECs possess several structural and regulatory roles, participating to the vascular homeostasis and vessel wall integrity. They are responsible for the maintaining of a selectively permeable and non-thrombogenic barrier, that regulates the smooth muscle cells tone through the secretion of vasoconstrictors and vasodilatators, they modulate the immune response through the expression of chemotactic and adhesion molecules. ECs are located on the basal lamina, composed of collagen type IV, proteoglycans, laminin and fibronectin. Regarding more specifically the abdominal aorta, the intima layer is mainly composed by type I collagen (16%), while type III and type IV collagen are present in a lower percentage. The sub-endothelial layer mainly contains smooth muscle cells and bundles of collagen fibrils. Collagen fibres are not arranged according to the same orientation through the whole layer, but many layer of collagen with a distinct pattern of orientation are present. (Gasser et al., 2006). The tunica intima terminates with an *internal elastic lamina*, which creates the limit with the medium layer of the artery wall. The internal elastic lamina changes according to the arterial type: it is very thin and hard to be distinguished in elastic arteries, whereas it is very prominent in muscular arteries.

The media is the middle and, in terms of mechanical properties, the most important layer of the aortic wall. The internal elastic lamina and the external elastic lamina separate the tunica media from the intima and adventitia, respectively. The media is composed by smooth muscle cells (vSMCs), organized as complex network of elastin and collagen, mainly type I, III, IV and V. An extracellular matrix (ECM), containing proteoglycans, surrounds the cellular and elastic components. vSMCs are spindle shaped, with an elongated nucleus and assume a specific orientation in elastic and muscular arteries. In elastic arteries, vSMCs are arranged into 5-15µm thick concentric structures, separated by elastin. At physiological distending pressures, the aortic smooth muscle, elastin and collagen are precisely organized into distinct layers, termed *lamellar unit* (Wolinsky and Glagov, 1964). In particular, according to the model of aortic media in

mammals proposed by Wolinsky and Glagov, elastin fibres are organized into fenestrated sheets, or lamellae, forming concentric layers, with finer elastin fibres that interconnect adjacent lamellae; bundles of collagen are interposed between elastin sheets, circumferentially oriented (Wolinsky and Glagov, 1964). In a later work, Clarke and Glagov defined the lamellar unit as the *"musculo-elastic fascicle"*, that constitutes both the structural and functional unit of the aortic media; the number as well as the orientation and the composition of these units are functional to the homogeneous distribution and magnitude of the tensile stress along the vessel wall (Clark and Glagov, 2015). Medial vSMCs are essential to the correct functionality of the vasculature; indeed, their contraction or relaxation modulate the arterial structural and diameter changes, adapting to the blood dynamics and keeping blood pressure. In addition, SMCs are responsible of the synthesis of ECM components, vSMCs are susceptible to the phenotype changes, shifting from contractile to synthetic, characterized on the basis of different cell shape and structure, marker expression, proliferative and migration rates. Contractile vSMCs show an elongated, spindle-shape morphology, substituted by a less elongated, epitheloid-like shape in synthetic vSMCs. The synthetic phenotype is characteristic for the presence of a high number of organelles appointed to protein synthesis, as well as for a high proliferative rate. In contractile vSMCs, biosynthetic organelles are replaced by contractile filaments; in addition, the contractile SMC phenotype presents increased contractile markers, such as alpha-smooth muscle actin, smoothelin, smooth muscle heavy chain, that are not represented in synthetic vSMC (Rensen et al., 2007). vSMC heterogeneity exists not only between different tissues and vascular districts, but also in the same vessel, suggesting a genetic basis. Moreover, vSMC phenotype changes in dearly development: during blood vessel formation, vSMCs assume a contractile phenotype, corresponding to a reduced ECM production and an increased myofilament formation, allowing the vessel contraction and dilation under the effects of blood flow and pressure. A reversal of vSMC phenotype, from contractile into synthetic, induces cell proliferation and invasion of the intima tunica, characteristic of vascular disorder. Some differences concerning the number of lamellar units, the elastic fibres composition and the nutrition mechanism have been observed between the thoracic and abdominal region of human aorta, thus, implying a different response to injuries and pathological processes. The number of lamellar units increases with arterial diameter and mechanical force; in addition, it defines the thickness of the aortic media and influences the presence of *vasa vasorum*, small vessels that arise from the adventitia and supply nutrients to the media. When the number of lamellar units is about 28-30 (0.5 mm thickness), as in smaller elastic arteries, the media receives nutrients through a simple diffusion mechanism from blood vessel lumen, traversing endothelial layer. Arteries with more than 28 units receive nutrient from vasa

vasorum. Human thoracic aorta contains 55-60 lamellar units, that increase in number through the synthesis of new additional lamellar units; human abdominal aorta, regardless its diameter, has 20-32 units, that undergo an expansion process without a numerical increase. This difference may explain the major elastin content in thoracic aorta than abdominal aorta; in addition, the elastin content was shown to decrease with age. A reduced number of lamellar units, a decreased elastin content and an inadequate nutrient transport create the structural conditions (loss of stiffness and recoil capacity of elastin) that predispose the abdominal aorta to an increased risk of aneurysm formation. (Wolinsky, 1970) (Ruddy et al., 2008). The media of muscular arteries has a less-defined structure, predominantly made of thick smooth muscle layer and an internal elastic lamina. Smooth muscle is arranged into concentric layers, whose number can range between 25 and 35 in larger arteries; these smooth muscle structures are surrounded by connective matrix that works as a substrate for the cell components and, meanwhile, contributing to the artery strength and force.

The *adventia* (tunica adventitia) is the outermost layer of the arterial wall, representing the 10% of elastic arteries and the 50% of muscular arteries. It is mainly composed of: fibroblast, macrophages, collagen and groudmatrix. The adventitia shows a various level of thickness, depending on the localization and function of the vessel wall. Type I collagen is the predominant component of the connective tissue and polarized light microscopy showed an organization into helical structures. The collagen is the main constituent and it contributes to the adventitial providing support and strengthen to the arterial wall, preventing the excessive distension of the vessels. Nerves and vasa vasorum are also characteristic of adventitial structures. Nerves contribute to the regulation of medial smooth muscle activity, through the release of neurotransmettitors, like norepinephrine and acetylcholine. Vasa vasorum represent an intra-vascular network of small vessels, including arterioles, venules and capillars, that can reach the media, as a nutritive support to the arteries whose thickness does not allow the nutrient delivery through the diffusion from the intima. The structural changes that affect the arterial wall is named *vascular remodeling*, which interests larger elastic arteries more than smaller and muscular ones. Ageing represents the main decisive factor for arterial remodeling, mainly characterized by an increased thickness of media and, most frequently, intima. In a young healthy subject, the intima is very thin; ageing can be responsible of connective tissue alterations, followed by an increase of collagen content. Moreover, endothelial cells can be influenced by ageing process both on the structural and functional hand. First, can change their morphology, acquiring a bigger size and an irregular shape; they can also loose most of their regulatory roles. Endothelial layer can become

more permeable, allowing the transit of many substances and vSMC infiltration. These alterations characterized the atherosclerotic process.

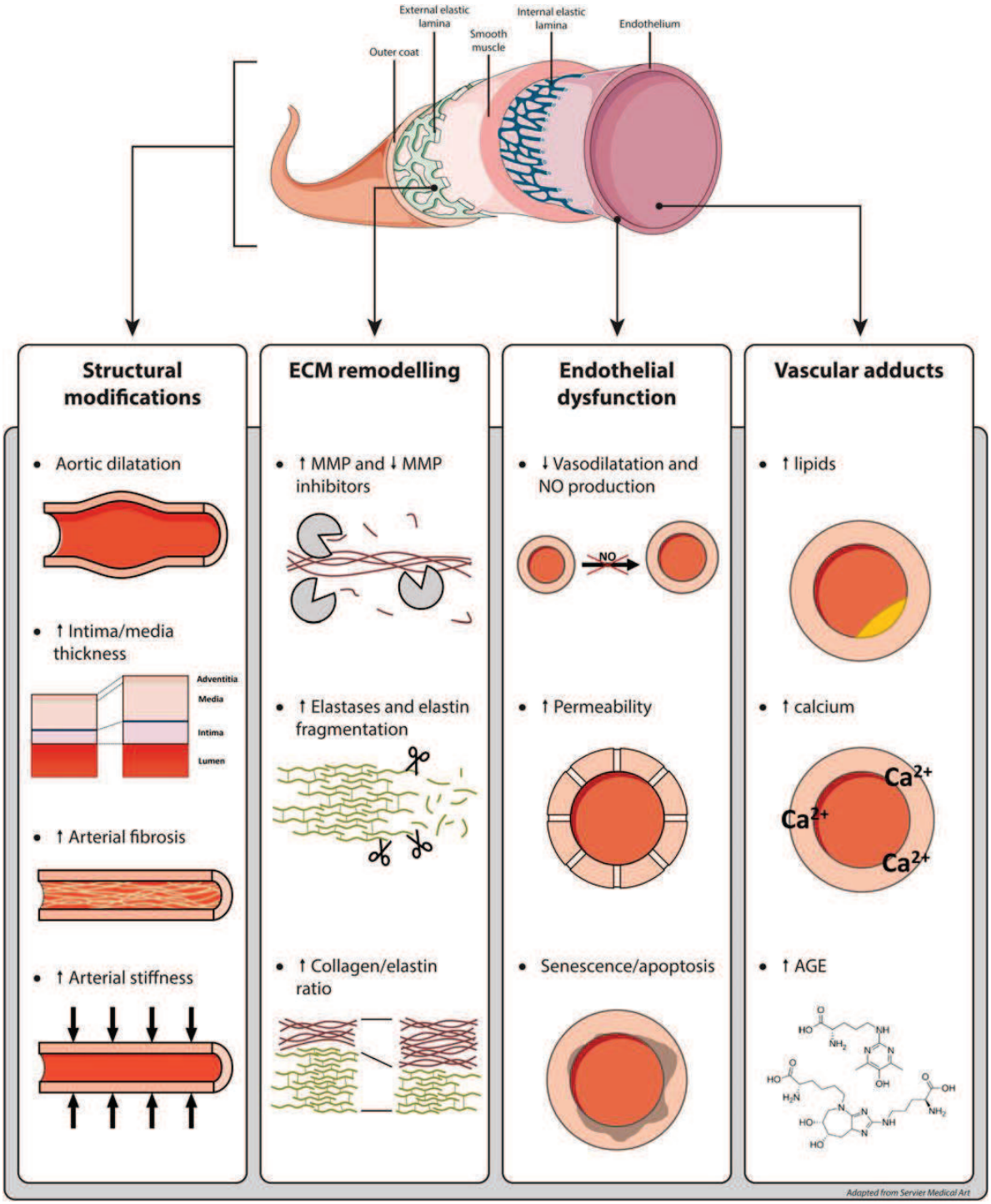


Figure 3. Molecular and cellular events occurring during vascular remodeling.

In aged arteries, all these events contribute to decrease compliance, resilience, and increase stiffness. ECM, extracellular matrix; MMP, matrix-metalloproteinases; NO, nitric oxide; AGE, advanced glycation end-products. (Duca et al., 2016)

1.1.3 Extracellular matrix in vascular tissues

Extracellular matrix (ECM) is synthesized by vSMCs and adventitial fibroblasts, and constitutes the scaffold that supports the structure and the function of the vessel wall, driving its biomechanical properties. ECM does not act only as a structural support, but also regulates many cell functions, including proliferation, migration, differentiation and morphological changes (Daley et al., 2008). The main ECM components are fibrous proteins that confer tensile strength and visco-elasticity to the wall, as well as proteins that contribute to ECM network building.

Collagen is a tense protein that prevents vessel wall excessive distension. About 26 members of this family have been identified and their expression and distribution depend on the cell type. Vascular cells, type I and type III collagen are the most common types, representing 30% and 60% of the vascular wall; the remaining 10% is composed type V, XII and XIV collagen (Wagenseil and Mecham, 2009). Structurally, the basic unit of collagen is given by a 300 nm triple helix. Each chain, called α-chain, contains 338 (type I) and 340 (type II) triplets of the aminosequence Gly-X-Y (generally, X is a proline and Y is an hydroxyproline). Post-trascriptional modifications are required before collagen maturation into a functional protein. Crosslinking Lysil-oxidase increases collagen insolubility and tensile strength by crosslinking. Lysyl oxidase (LOX) is a copper-dependent amine oxidase that initiates the covalent cross-linking of collagen and elastin. LOX catalyses an oxidative de-amination of lysine and hydoxylysine residues to peptidyl a-aminoadipicd-semialdehydes. These highly reactive semialdehydes can spontaneously condense to form intra-and intermolecular covalent cross-linkages that assure ECM stability. Strong evidence about the involvement of a reduction of LOX activity in the pathogenesis of AAA is available. (Rodríguez et al., 2008)

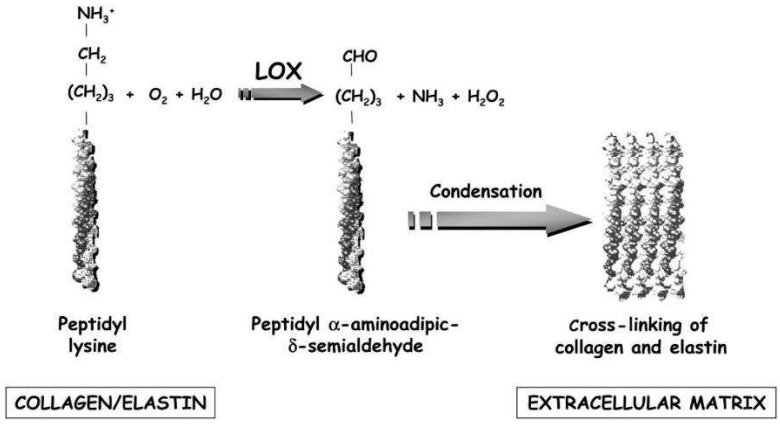


Figure 4. Reaction catalyse by LOX. LOX oxidizes primary amines on collagen and elastin substrates to reactive semialdehydes that form by condense covalent cross-linkages. (Rodríguez et al., 2008)

Blood vessels are endowed with the property of viscoelasticity, which allows to buffer pressure variations during the cardiac cycle, permitting an adequately constant blood flow and correct organ perfusion. This mechanical phenomenon is due to the presence of highly resilient elastic fibres. They are arranged in concentric fenestrated elastic lamellae. Each elastic lamella alternates and it is physically connected with a concentric ring of vSMC forming the lamellar unit. Mature elastic fibres and lamellae are comprised of a homogenous core containing elastin that is assembled along parallel microfibrils. Microfibrils are composed of numerous heterogeneous glycoproteins, such as fibrillin-1 and fibrillin-2, microfibrin-associated glycoproteins (MAGP1 and MAGP2) and the latent transforming growth factor β (TGF- β)-binding proteins (Kielty et al., 2002). Microfibrils interact with other proteins localised to the elastin-microfibril interface or to the cell surface-elastic fibre interface called elastin microfibril interface-located proteins (EMILINs) and fibulins. However, elastin is the most prominent component of the large arteries wall.

Elastin gene is located on chromosome 7 and its expression is high during pre- and neonatal development (Arribas et al., 2006). In humans, its activity is restricted to the early life, and the mature form of elastin has a half-life of 40 years. Elastin degradation is related to aging and diseases. Elastin gene encodes for a precursor form of the functional protein, named tropoelastin. This is a monomeric protein (64-72 kDa), whose structure is characterized by the alternans of hydrophobic domains and lysine residues with cross-linking motifs. When tropoelastin is released in the extracellular space, lysine residues undergo modifications to form covalent binds with elastin molecules. As collagen, crosslinking is mediated by lysil-oxidase and takes to sequential reactions leading to the functional and insoluble form of elastin. Unlike elastin that has 15-20 cross-links per unit, collagen only contains 1-4 cross-links. The high number is critical for elastin recoil property, as well as for insolubility and high half-life (Eyre et al., 1984). As reported before, under native conditions elastic fibres have short period of active synthesis, restricted to fetal life and neonatal period. During this period, tropoelastin is continuously produced and assembled into extracellular elastic fibres. Afterwards, tropoelastin is not actively synthesized in blood vessels. The first prerequisite step in elastogenesis involves assembly of microfibrils close to the cell surface, probably modulated by the cell surface integrins and heparin sulfate proteoglycans. It requires proteolytic modifications of the secreted fibrillins before the association with MAGPs and other microfibrillar components. The hydrophobic and non-glycosylated tropoelastin requires to be chaperoned through the intracellular secretory pathways. In the endosomal and Golgi compartments, tropoelastin is escorted by a 67 kDa elastin binding protein (EBP) that protects from premature intracellular self-aggregation and degradation by

serine proteinases. This interaction triggers the release of newly secreted tropoelastin from the chaperone and their subsequent orderly binding to the microfibrillar scaffold. This systematic binding secures a proper mutual orientation of the adjacent tropoelastin molecules and allow for the process of ordered self-assembly (coarcevation). Coarcevation allows for the proximity of lysine residues (located in "cross-linking domains") that can be covalently cross-linked together after their oxidative deamination by a LOX (Kagan and Li, 2003) to form linkages called desmosines and isodesmosine. The fully cross-linked elastin turns into an "insoluble" polymer. Vascular elastin is produced by SMCs in the media and fibroblasts in the adventitia, but also endothelial cells display some limited elastogenic abilities. Elastin confers elasticity to the vessel wall, the most essential property in large diameter arteries which are subjected to high pressures generated by heart beating and blood flow, defining their ability to contract and distend according to the cardiac cycle. In addition, elastin and elastin-derived peptides (EDP) have been shown to regulate cell proliferation and phenotype.

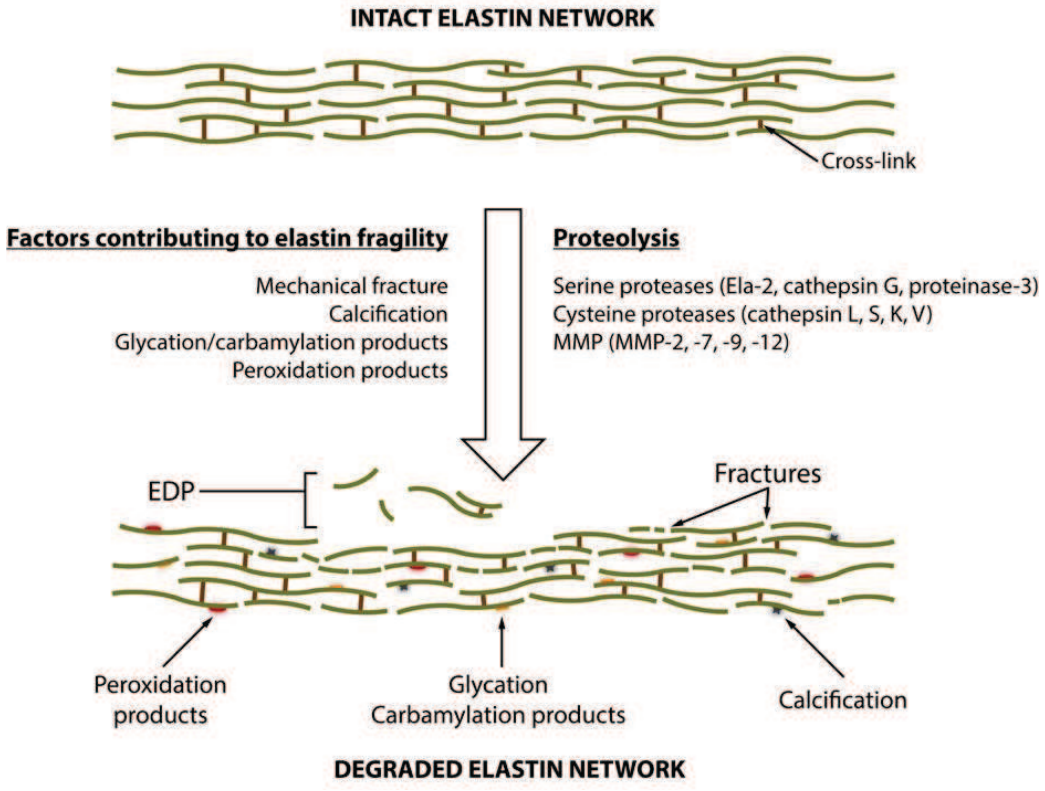


Figure 5. Elastin remodeling.

During ageing, elastin is subject to remodeling and fragmentation. Different factor such as mechanical fracture, calcification, glycation, carbamylation and peroxidation can contribute to elastin fragility and rupture. In addition, these factors enhance elastin susceptibility to proteolysis by specific elastases such as serine, cysteine, and MMPs. (Duca et al., 2016)

Elastokines are the bioactive EDP generated by the action of elastases and, all elastokines act by binding to a singular cell surface receptor named the elastin receptor complex (ERC). ERC is derived from the lysosomal β-galactosidase (β-Gal) complex and is expressed at the plasma membrane of numerous cell types (Duca et al., 2004). It is a heterotrimer composed of a peripheral 67 kDa subunit noted elastin-binding protein (EBP), which binds elastin peptides, a 55 kDa protective protein or cathepsin A (PPCA), and a 61 kDa membrane-bound neuraminidase, Neu-1 (Hinek et al., 2006). From a mechanistic point of view, when an elastin peptide binds to EBP, it is recruited to the cell membrane where it interacts with PPCA and Neu-1 to form the functional ERC enhancing diverse signaling pathways. Depending on the cell type, various signaling modules are triggered, converging to the activation of ERK1/2, which appear as crucial actors in elastin peptide signaling. In smooth muscle cells, EBP-induced proliferation involves activation of Gi proteins concomitantly with opening of Ca^{2+} L-type channels. Finally, in endothelial cells, a PI3Kγ/Akt/eNOS/NO/PKG1 module is involved in ERK1/2 activation (Fahem et al., 2008).

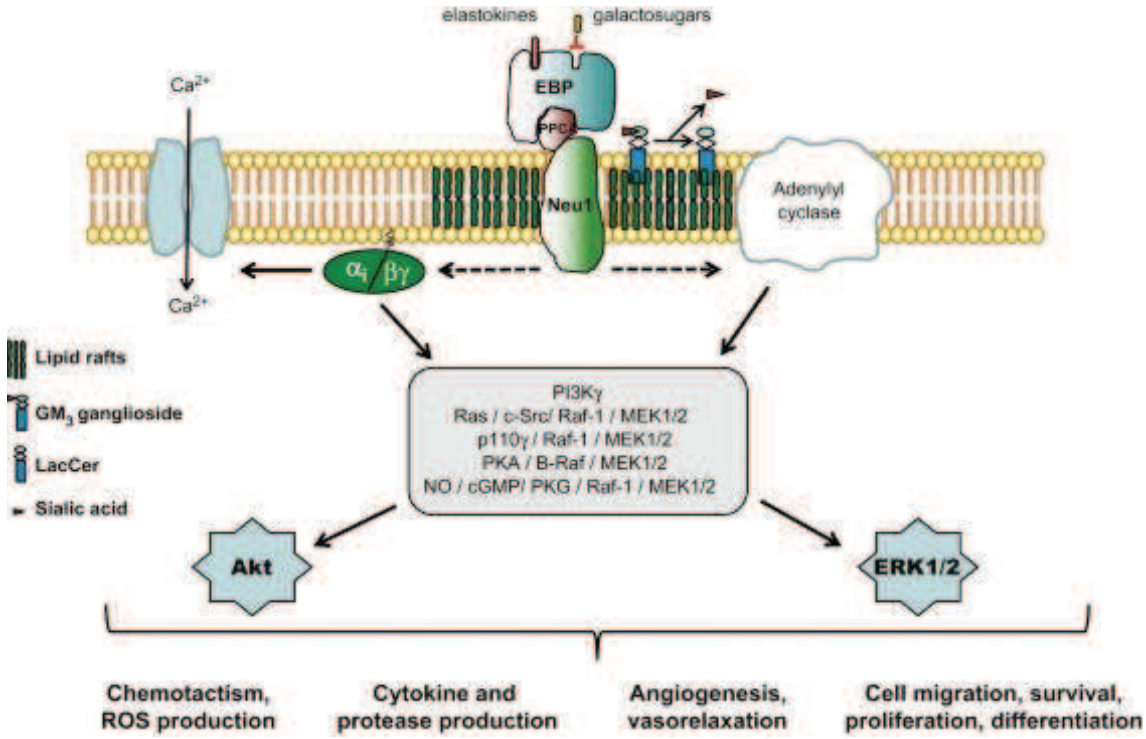


Figure 6. Elastokines signaling.

Elastin receptor complex (ERC)-induced signaling pathways and associated cellular response. cGMP, cyclic guanosine monophosphate; EBP, elastin-binding protein; ERK1/2, extracellular signal-regulated kinase 1/2; MEK1/2, mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2; NO, nitric oxyde; PI3Kγ, phosphoinositide-3 kinase γ; PKG, protein kinase G. (Maurice et al., 2013)

1.1.4 Biomechanical properties of human arterial wall

Vessel wall are continuously exposed to a wide range of hemodynamic forces. Because of the pulsatile nature of blood and the complexity of the arterial system, being composed of various size vessels, branches and bifurcations, the fluid mechanic laws cannot be simply applied to explain these mechanics (Resnick et al., 2003). Blood vessels are constantly subjected to various types of hemodynamic forces, including hydrostatic pressure, cyclic stretch, and fluid shear stress. Shear stress (τ) is parallel to the vessel wall and represents the frictional force that blood flow exerts mainly on the endothelial layer. Instead, cyclic stretch (ρ) is the stress perpendicular to the vessel wall and represents the circumferential deformation of the blood vessel wall during distension and relaxation of the recurring cardiac cycle.

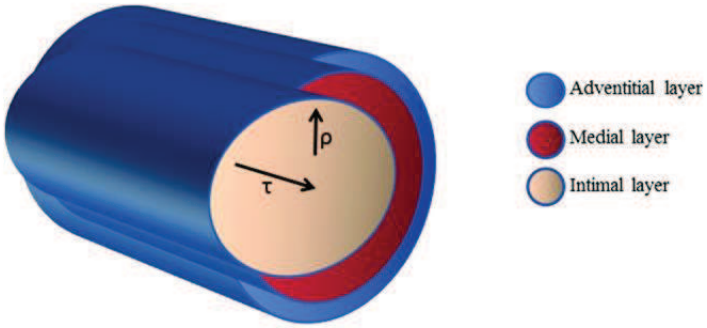


Figure 7. Mechanical and hemodynamic forces associated with blood flow.
Shear stress (τ) is parallel to the vessel wall and represents the frictional force that blood flow exerts mainly on the endothelial surface of the vessel wall. Instead, cyclic stretch (ρ) is the stress perpendicular to the vessel wall and represents the circumferential deformation of the blood vessel wall during distension and relaxation of the recurring cardiac cycle.

Hemodynamic forces associated with blood flow play a central role in the homeostasis of the circulatory system. These forces act on the vascular wall, contributing to the regulation of myogenic tone, responses to vasoactive molecules, gene regulation, vascular permeability and remodeling. Hemodynamic loading breakdown, can be responsible for the development of vascular diseases, including hypertension, thrombosis, aneurysms and atherosclerosis. While physiological cyclic stretch induces cell cycle arrest in VSMC, chronically increased blood pressure and vascular transmural stress activate vascular cell proliferation (leading to luminal stenosis), collagen and fibronectin synthesis which results in thickening of the vascular wall as a feature of hypertension-induced vascular remodeling eventually altering arteries compliance. (Hipper and Isenberg, 2000)(O'Callaghan and Williams, 2000)

Although vascular endothelial cells and vascular smooth muscle cells are exposed to both types of mechanical forces, shear stress resulting from blood flow is sensed mainly by ECs

(Davies, 1995), whereas both ECs and SMCs are subjected to cyclic stretch resulting from pulsatile pressure. Vascular cells have with numerous receptors that sense external stimuli such as strain, pressure and fluid shear stress. These receptors transduce the mechanical signals into a biological response through a process named mechanotransduction. The cytoskeleton and other structural components have an established role in mechanotransduction, being able to transmit and modulate tension within the cell. SMCs and ECs are able to perceive stretch and shear stress alterations through a system of receptors, that include integrins, G-protein receptor, ion channels, adhesion molecules and activate a signal transduction, also involving extracellular matrix (Resnick et al., 2003) (Lehoux et al., 2006).

As previously described, endothelium works as a selectively permeable barrier that regulates the macromolecules entrance from the blood into the vessel wall (i.e. low-density lipoprotein, LDL). In this view, endothelium has a protective function, together with an anti-thrombogenic property; moreover, it guides the inflammatory process and drives the SMC contraction through the release of vasocostrictors and vasodilatators. Any impairment in these activities is crucial for the development of pathological processes, especially related to proatherogenic and pro-thrombotic events. Each shear stress variation is sensed by ECs, that undergo several mechanisms of adaptation to acute as well as chronic changes. One of EC characteristics consists in the alignment according to the blood flow. Under physiologic condition, the mean intensity of shear stress is typically of 10-15 dynes/cm^2 in large arteries. (Tsou et al., 2008) ECs with an elongated shape and aligned with blood flow reflect a laminar and unidirectional flow; closely to side branches, bifurcations and curvatures, the flow becomes turbulent, creating secondary flows with different directions as well as recirculation sites (vortices); in this case, ECs assume a less oriented configuration, a polygonal shape and this morphological alteration is mediated by the cytoskeleton and microtubules rearrangement.

The introduction of DNA microarray technique allowed the identification of a great quantity of genes regulated by shear stress, highlighting many molecular mechanisms involved in vascular wall pathophysiology. Interestingly, genes encoding for anti-oxidant factors, ECM proteins, growth arrest and gap junction proteins were shown to be induced under chronic laminar shear stress, revealing an atheroprotective role; conversely, proatherogenic and pro-inflammatory genes were stimulated in response to an altered shear stress (Malek et al., 1999) (Li et al., 2005). Although shear stress mainly acts on ECs, it can influence SMC phenotype and alignment, in mechanism mediated by ECs. According to SMC culture in an *in vitro* two-dimensional level, a laminar shear stress stimulates the acquisition of a contractile phenotype, increases SMC

apoptosis mediated by EC release of NO and reduces the cell proliferation (Sterpetti et al., 1993), inducing the cell-cycle arrest. Conversely, an oscillatory flow takes to SMC proliferation and migration, in a MMP-dependent mechanism, characteristics of the synthetic phenotype. Interestingly, SMC proliferation was observed closely to atherosclerotic sites, with high shear stress (Yoshida et al., 1990). Differently from shear stress, mechanical stretch directly affects SMCs, both at the structural and molecular level. At physiological strain (10%, 1 Hz), SMC proliferation resulted inhibited through different mechanism, one of which involves the G1/S arrest during cell cycle. All these data are concurrent with the belief that SMC under physiological stretch are differentiated and reveal a contractile phenotype. Under hypertensive condition, SMCs are exposed to high level of stretch, due to the increased blood pressure and, according to Laplace's equations (T=Pr/h), increased proliferation and enhanced synthesis of collagen and elastin (Lehoux et al., 2006).

From a molecular point of view, SMCs express many different receptors and ion channels, which can be activated by mechanical stress leading to the activation of multiple classic signaling pathways, such as G protein, kinases, calcium, cAMP, NO, eNOS, MAPK and MKP-1. Moreover, mechanical stresses appeared simultaneously to initiate multiple signaling pathways. Mechanical stresses stimulate conformational activation of cell integrins and increase cell binding to extracellular matrix. The dynamic formation of new ligand-integrin connections is required for stretch induced mechanotransduction. During the stimulation of vascular cells by mechanical factors such stretch, several signaling events are associated with the formation of focal adhesion, which comprise integrin cluster and cytoskeletal protein. The proteins present at focal adhesion become phosphorylated on tyrosine when the cells are stimulated, a FAK activation is an indicator in focal adhesion formation (Boccafoschi et al., 2011)(Lehoux et al., 2006).

Once cells adhere to ECM proteins, integrins became activated and form cluster at the cell surface thus initiating the formation of adhesion complexes which will enhance FAK's catalytic activity and increase FAK tyrosine phosphorylation. FAK phosphorylation at tyrosine 397 (Y397) leads to the recruitment of Src and Src-family kinases as well as to an increased phosphorylation of other proteins present in the adhesion complex such as paxilin and p130Cos. (Arias-Salgado et al., 2003) (Lehoux et al., 2005) Subsequent phosphorylation of specific tyrosine residues leads to the recruitment of additional SH2-domain-containing signaling proteins such as PI3K and Grb2.43 Among integrin receptors, integrin α5β3 has been identified as having particularly interesting expression pattern among the vascular cells during angiogenesis and vascular remodeling. In particular, integrin α5β3 binds numerous ECM ligands with exposed RGD peptide

as fibronectin, vitronectin, fibrinogen and it is involved in the response to mechanical strain. In the '90s, Wilson and colleagues established a link between the mechanical stimuli and the action of growth factors. Their studies demonstrated that cyclic stretch increased VSMC proliferation when plated on collagen, fibronectin or vitronectin, but not on laminin or elastin; further, these effects required the production of PDGF suggesting a tight interaction between integrins and growth factors downstream signaling pathways related to mechanical stimuli. Generally, growth factors receptors belong to two classes of membrane proteins: tyrosine kinase receptors and G-coupled receptors and G proteins. Another membrane structurse involved in mechanosensing are ion channels. VSMCs stimulated by mechanical stress result in a transient increase in intracellular calcium and divalent cations and depolarization which maintain smooth muscle tension. Opening these channels causes Ca^{2+} and Na^{+} influx and membrane depolarization, which contributes to the myogenic response to mechanical stretch. (Marrero et al., 1996)

The altered stretch-activated channels in arterial SMCs may contribute to the enhanced myogenetic responses as well as the generation of hypertrophy and remodeling of arterial tissue in hypertension. All the cited membrane mechanosensors have a common intracellular pathway converging to MAP kinase signaling. MAP kinase comprises a ubiquitous family of threonine/tyrosine kinases, and includes extracellular signal-regulated kinases (ERK), stress activated protein kinases (SAPK) or c-Jun NH2-terminal kinases (JNK) and p38/ERK kinases. (Karin, 1998) This cascade is an important pathway whereby signal originating from mechanical forces can lead to gene expression and protein synthesis in vSMC.

1.2 Abdominal Aortic Aneurysm

1.2.1 Definition, epidemiology and risk factors

The term "aneurysm" is used to define a permanent focal dilatation of the vessel wall. Mc Gregor et al. defined abdominal aortic aneurysm (AAA) as an aortic segment with a diameter larger than 3cm (McGregor et al., 1975); following this definition, aneurysms of the infrarenal, intrarenal and suprarenal aorta are included (Sakalihasan et al., 2005). Aneurysms can be distinguished into fusiform and saccular, according as the whole aortic circumference, or only a small segment of the aortic circumference, is interested.

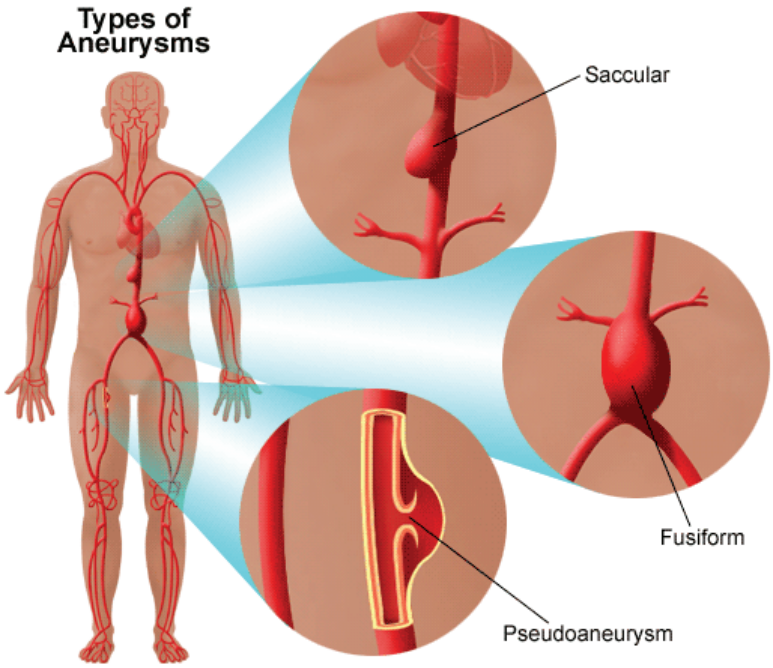


Figure 8. Type of aneurysms (stanfordhealthcare.org)

AAAs comprise the 15th leading cause of death among men over 65 and result in more than 15,000 surgical procedures annually in the United States. Although prevalence of aneurysms is increasing the mechanisms that regulate aortic aneurysm development remain almost undefined (Lederle et al., 2000) (Salmon et al., 2013). Approximately 80% of all aortic aneurysms occurs below the renal arteries, and the majority has an atherosclerotic cause. Other less common causes include inflammatory aneurysm, tuberculous aneurysm and syphilitic or infectious aneurysm.

It usually remains asymptomatic until the rupture occurs. Because of the ageing of the population, the increasing of smokers and the improved screening and diagnostic tools AAA incidence is recently increased. Major risk factors for the occurrence of AAA are male gender,

age, smoking, race, pre-existent cardiovascular affections, such as hypertension and atherosclerosis. The risk of AAAs increases dramatically after 60 years of age (Singh, 2001). Clinically relevant aneurysms (more than 4 cm in diameter) are present in approximately 1% of men between 55 and 64 years of age, and the prevalence increases by 2% to 4% per decade thereafter (Singh, 2001). AAAs are four to six times more common in men than in women (Scott et al., 1995) (Lederle et al., 2001). Moreover, AAAs develop in women approximately 10 years later than in men (McFarlane, 1991). The related mechanisms of sex differences are not fully understood, although women seem to be protected by female sex hormones (Makrygiannis et al., 2014).

AAAs were found to occur more frequently in Caucasian population than in Afro-American ethnicity. Due to its promoting effects on inflammation, proteolysis, and smooth muscle cell (SMC) apoptosis, smoking is another strong risk factor for the development of AAAs. (Li and Dai, 2012). In addition to these environmental components, genetic aspects play an important role. A positive familiar history for AAA, especially in male first-degree relatives, is associated with an increased risk. Rupture of an AAA and its associated catastrophic physiological insult carry the overall mortality more than 80%, and 2% of all deaths are AAA-relate (Nordon et al., 2011).

Most aneurysms are small in size and they do not need the surgical repair, but the increase in the aortic diameter can lead to rupture, which can be fatal. Medical therapy may be helpful in patients with small- to medium-sized aneurysms not elective for surgery (thoracic and abdominal aortic aneurysms). Appropriate and immediate therapy is essential with the aim of stabilizing the patient and improving the clinical outcome. Therapy's targets are blood pressure control, decrease of shear stress, optimization of anticoagulation, volume management, and pain control. Different pharmacological treatments are available: beta-blockers, tetracycline, statins and angiotensin-converting enzyme Inhibitors/Angiotensin Receptor Blockers. β-blockers may be beneficial for reducing the rate of aortic dilatation. This is thought to be due to the effect of β-blockers in reducing left ventricular dP/dt and reducing shear stress. In addition, β-blockers reduce dP/dt in the aorta and might be beneficial via this mechanism and the resultant effect on shear stress in the aorta (Danyi et al., 2011). Although data regarding therapeutic benefit of beta-blockers in management of AAA are limited, beta-blockers have been shown to significantly reduce the expansion rate of AAA. The 2005 ACC/AHA guidelines recommended beta-blocker therapy in patients with an AAA not elective for surgery. Doxycycline is a nonspecific MMPs inhibitor (Petrinec et al., 1996). This antibiotic has been used in conditions with MMPs overexpression

(e.g., periodontal disease, rheumatoid arthritis) (Hanemaaijer et al., 1997). In animal models, doxycycline slowed elastin degradation and aneurysm development (Xiong et al., 2008). In human, doxycycline decreased MMP-9 levels (Baxter et al., 2002) and slowed the rate of progression of AAA. Statin treatment is one of the elective therapies in cardiovascular diseases. Statins reduce the progression of atherosclerosis and improve clinical outcomes. In addition to their lipoprotein-reducing properties, statins have pleiotropic effects. For instance, they reduce oxidative stress by blocking the effects of reactive oxygen species on aneurysms. This effect is independent of their lipid-lowering properties. Statins achieve these results through suppressing the NADH/NADPH oxidase system (Ejiri et al., 2003). Angiotensin II has been shown to have several biological effects on the cardiovascular system. It promotes vascular hypertrophy, cell proliferation, production of extracellular matrix, and activation of macrophages, and it activates NADH/NADPH oxidase of vascular smooth muscle cells. Angiotensin-converting enzyme inhibitors (ACEIs) have been shown to both stimulate and inhibit MMPs and the degradation of extracellular matrix in aortic aneurysms (Rizzoni et al., 2003).

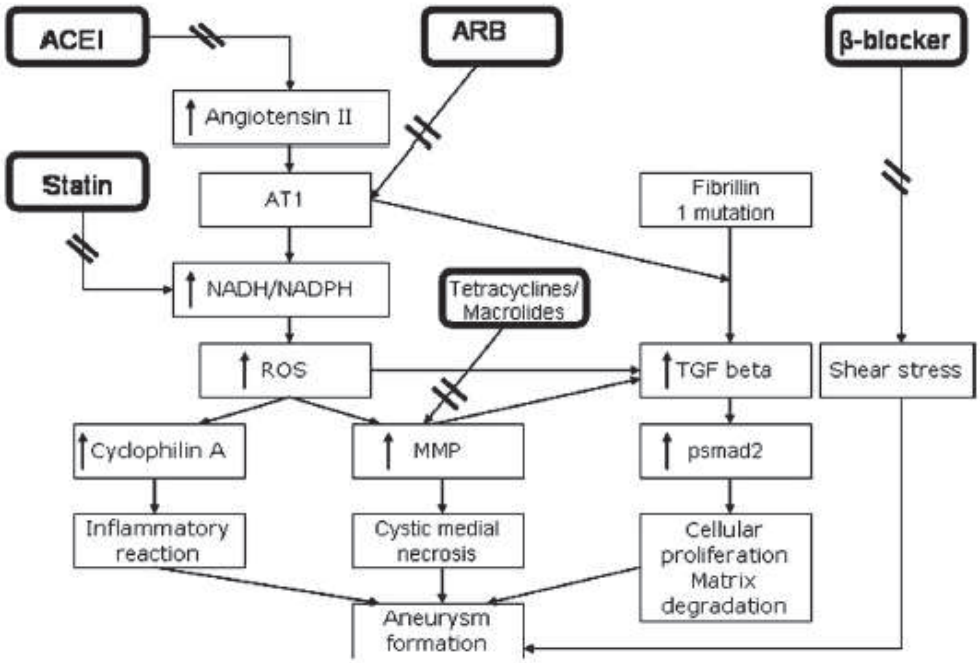


Figure 9. Molecular mechanisms of aneurysm formation and the effects of different medications. Angiotensin promotes aneurysm formation through angiotensin 1 (AT1) receptors, increasing ROS via NADH/NADPH system. This promotes inflammatory reaction and subsequent medial degeneration, leading to AAA. Fibrillin gene mutations cause enhanced transforming growth factor (TGF)-β signaling, causing cell proliferation and MMP synthesis. Angiotensin-converting enzyme inhibitors (ACEIs) block angiotensin II. Statins block the NADH/NADPH system; tetracyclines and macrolides reduce MMP activity. β-Blockers reduce shear stress on the vessel. (Danyi et al., 2011)

1.2.2 Symptoms, diagnosis and treatment

Differently from inflammatory AAAs, non-inflammatory aneurysms are generally asymptomatic: only in 8-18% of AAA cases, patients reported symptoms (i.e. pain that radiates to back or legs or low blood pressure). For this reason, AAA are accidentally diagnosed, after clinical investigation in presence of other cardiovascular disease or during screening test. In order to confirm a diagnosis of AAA, a complete medical history and physical examination is performed in combination with instrumental diagnostic procedures such as: X-ray, computed tomography scan (CT-scan) and magnetic resonance imaging scan (MRI).

Conversely, rupture of aneurysm is accompanied by abdominal pain, hemorrhagic and a pulsatile abdominal mass. Aneurysm evolution and possible complications are generally evaluated by diameter measures, which represent the major index of aortic rupture risk. The use of non-invasive techniques detecting circulating levels of degradation peptides or proteins that actively participate to AAA molecular pathogenesis would be more useful and accurate to decide drug therapy or surgical intervention. About 30% of asymptomatic aneurysms are recognized by physical palpation as a pulsatile abdominal mass, but this type of inspection bears on inter-operator variability. Preferential diagnostic is represented by ultrasonography (UT), also applied to follow-up, surveillance of asymptomatic aneurysms and screening. UT has a high sensitivity and sensibility, without high costs. When surgical procedures are needed, CT is performed. It can also be helpful to define the best surgical approach. CT gives more detailed information about the aortic diameter, the shape of the aneurysm, the thickness of intraluminal thrombus, together with the presence of entrapped blood. In presence of an inflammatory AAA (IAAA), CT also indicates the extent of the inflammation. MRI is an alternative and more accurate diagnostic procedure, but it requires quite expensive cost.

The treatment choice depends on the aortic size and diameter (Sakalihasan et al., 2005):

· aortic diameter less than 5 cm: follow-up and surveillance through UT;

· aortic diameter between 5 and 5.5 cm: follow-up or surgery (the latter in case of female patient, familial history, high serum markers such as, plasma fibrinogen, D-dimer and IL-6)

· aortic diameter larger than 5.5 cm: surgery.

The goal for abdominal aortic aneurysm is to prevent rupture by controlling wall dilatation and weakening. Two different treatments for AAA are reported: open repair or endovascular repair.

Open surgical repair (OSR) was first reported in 1962 and still remains the treatment with the best long-term results. It is a major surgical procedure done under general anaesthesia, usually consisting of a midline laparotomy and cross-clamping of the aorta and iliac vessels. OSR is followed by the removal of the dysfunctional aortic segment and the insertion of a graft as substitute (DuBost et al., 1952). The mortality rate for OSR is between 3% and 7% and increases significantly in patients with comorbidities, particularly with coronary and carotid artery disease.

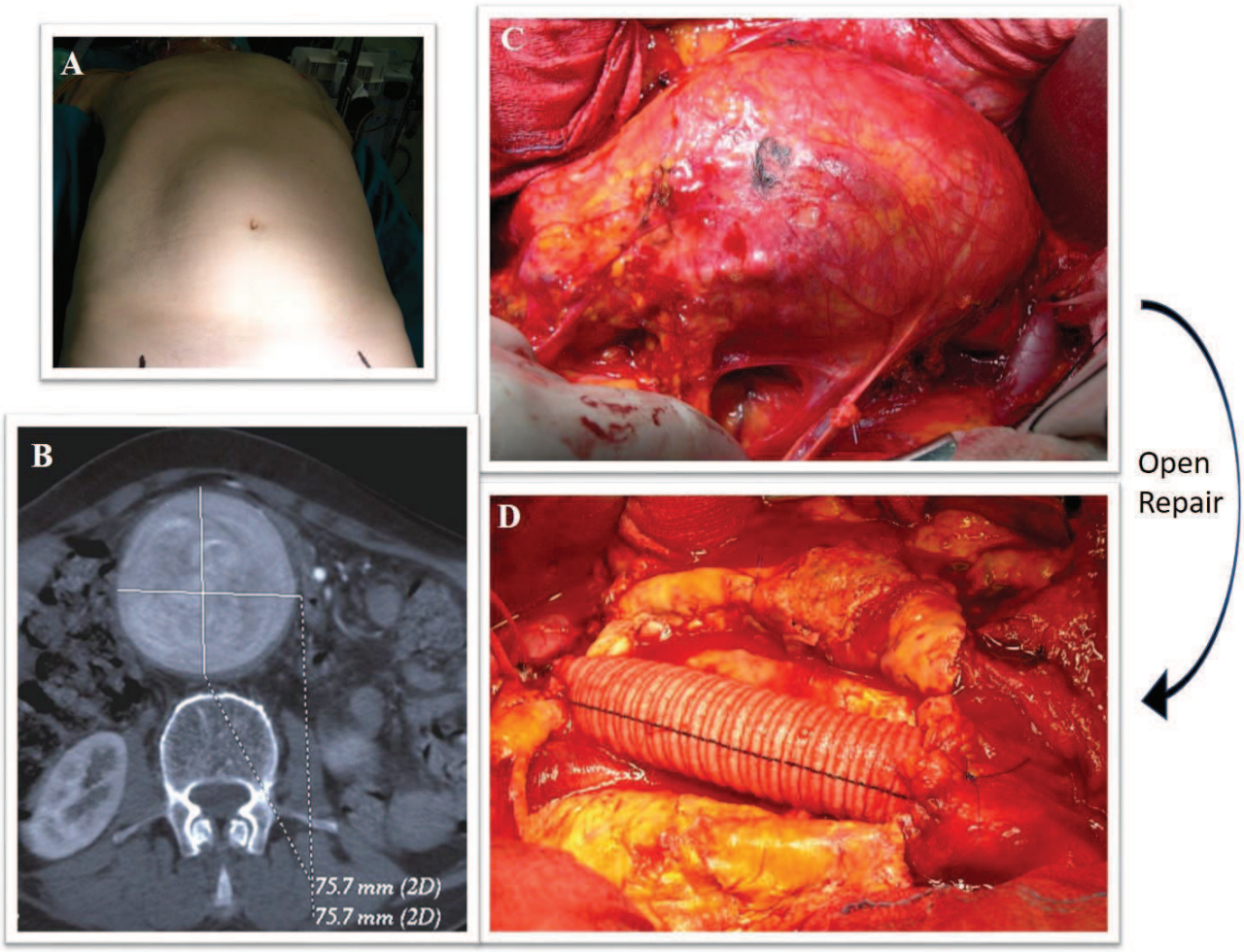


Figure 10. Open surgical repair (A) Abdomen of AAA patient (B) Abdominal CT showing aorta dilatation C) Aneurysm sac during surgical open repair D) Abdominal aortic graft in Dacron. Courtesy of Dr. Casella

The development of Endovascular Aneurysm Repair (EVAR) opens the way for a safer management of AAA. The long-term results of EVAR are comparable to those of OSR. EVAR was first described by Parodi in 1991. (Parodi et al., 1991) Juan Parodi implanted the first endograft in a human body and EVAR has become a milestone in the treatment of AAA, especially in presence of significant comorbidities. EVAR consists of the placement of a tubular graft within the aneurysm sac, using a metallic stent to fix it to the normal aortic and iliac wall.

The graft acts like an artificial blood vessel, excluding the damaged aortic segment from the normal blood flow, and thus, preventing the aneurysm progression and rupture. This reparative alternative does not require the surgical incision of the abdomen, thus avoiding complications consequent to the open surgery; in addition, it allows a shorter recovery time, reduced hospital stay and implies a lesser blood loss improving the patient quality of life. (Paravastu et al., 2014)

Several devices are available to treat AAA, differing with respect to design, modularity, metallic composition and structure of the stent, thickness, porosity and possible presence of an active method of fixing the device to the aortic wall. Appropriately-sized aortic endograft should be selected in relation to patient anatomy. The ideal characteristics are:

- ✓ Stent-graft size ranging
- ✓ Long durability
- ✓ Good biocompatibility
- ✓ Device delivery flexibility
- ✓ Radial force stability
- ✓ Low overall costs

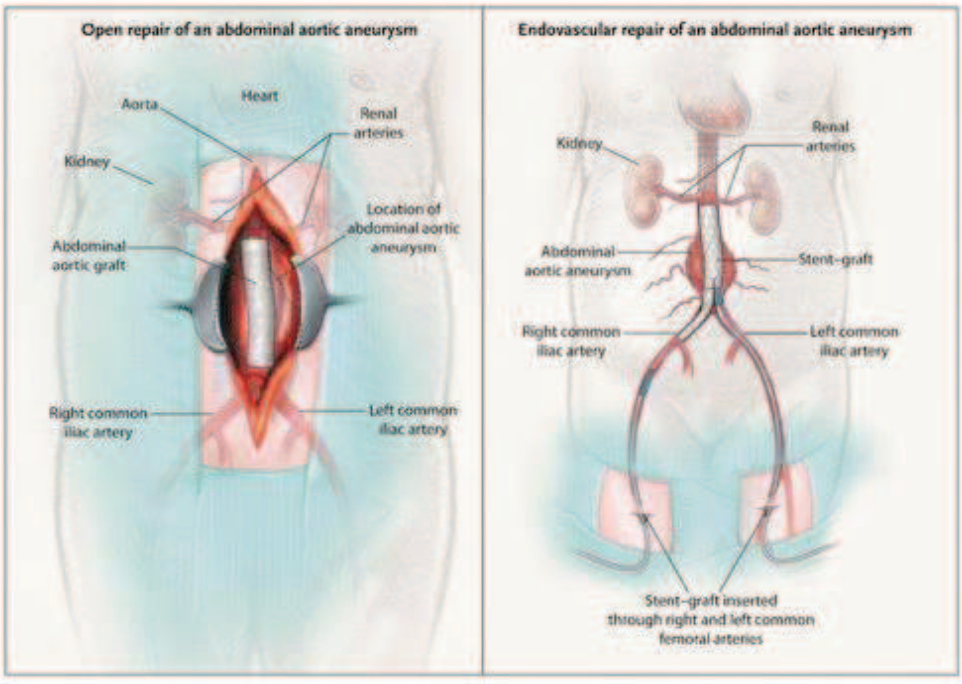


Figure 11. Open surgical repair (A) and Endovascular repair (B). (Schermerhorn et al., 2008)

One of the most frequent cause of EVAR failure is the “endoleak”, which happens when the blood flows into the aneurysm sac, external to the graft, and this condition can lead to

aneurysm expansion and rupture. Endoleak can be detected by angiography, CT scan or duplex ultrasound imaging and can be classified according to the timing of development. Primary endoleak (early endoleak) includes all cases that occur during the 30 days' perioperative period; secondary endoleak (late endoleak) represents a late complication of the correct seal (White et al., 1997). Endoleaks are further distinguished into five different types: *type I*, occurs when there is persistent blood flow around the proximal end of the graft or around the distal aspect of the graft; *type II* is caused from back-bleeding from side branches (inferior mesenteric artery and lumbar arteries) that retrograde fill the aneurysm sac; *type III*, derived from a mechanical failure or a structural defect of the graft; *type IV*, consequent to a graft wall porosity; *type V*, often referred to as "endotension," it happens when the aneurysm sac becomes pressurized without an identifiable source. (White et al., 1998)(Baum et al., 2003)

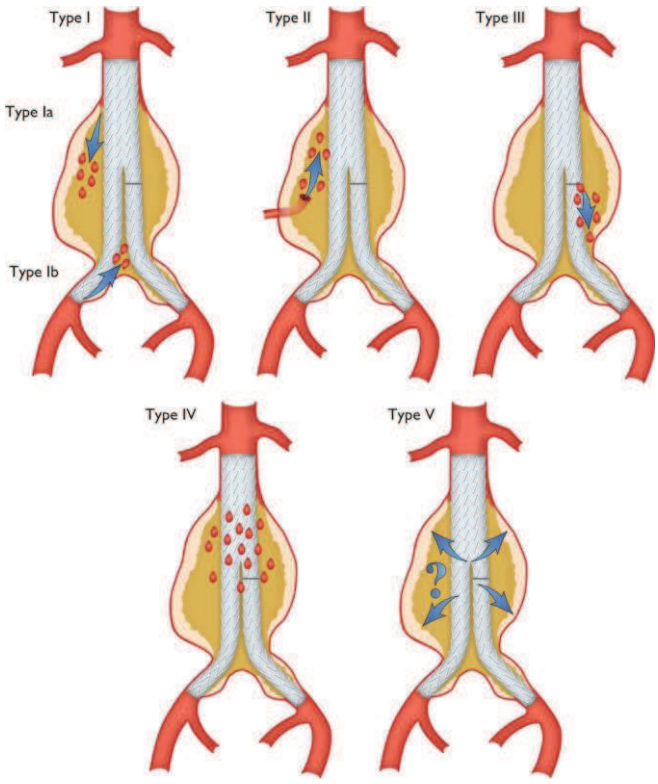


Figure 12. Classification of endoleaks.

(https://academic.oup.com/eurheartj/article/35/41/2873/407693/2014-ESC-Guidelines-on-the-diagnosis-and-treatment**)**

Although many researchers suggest that aneurysm formation is due to a combination of genetic, environmental and immune factors (Sakalihasan et al., 2005), the most common form of this dilatative affection rises consequently to atherosclerotic plaque disease, except in specific

cases of aneurysms associated with inflammatory diseases, connective tissue affections or traumatic event. The theory on the atherosclerotic origin of AAA has been based on the presence of atherosclerosis pathogenesis features in patients affected by aortic aneurysm. Current investigations and data about the specific nature of this relationship are still controversial; in addition, the aetiology and the key mechanisms involved in aneurysm initiation and expansion have not been completely elucidated. However, common features of aortic aneurysms are inflammation, matrix degradation and smooth muscle cells depletion. According to the most established theory, the "atherosclerotic aneurysm" develops as a complication to the pathological processes deriving from the atherosclerotic plaque development: matrix remodeling, thrombus formation and release of pro-inflammatory cytokines. An alternative theory considers aneurysm and atherosclerosis as independent events, that result from the same environmental and genetic conditions, but different dynamics are involved. In any case, according to current data describing the type of association between the two arterial affections, the majority of AAA are associated with atherosclerosis and elucidating this process would be crucial to develop more specific and targeted therapies. In the following table 1, major features of both atherosclerosis and AAA are listed.

	Atherosclerosis	AAA
Definition	Disease of the wall of large and medium sized arteries driven by hypercholesterolemia and subsequent aortic wall inflammation	Permanent localized dilatation of abdominal aorta that exceeds normal diameter by 50% accompanied by inflammatory response in the wall of abdominal aorta
Affected area	Large and medium size arteries	Abdominal aorta
Key risk factors	Age, obesity, dyslipidemia, hypertension, smoking, diabetes	Age, male sex, smoking (85% of patients), genetics, hypertension, obesity
Location of inflammatory infiltrate	Various immune cells accumulate in intima (internal layer of aorta) and adventitia (external layer)	Different subsets of immune cells accumulate in media and adventitia
Accumulating inflammatory cells	Monocytes, macrophages, dendritic cells, T cells, B cells, foam cells	Monocytes, macrophages, neutrophils, dendritic cells, NK cells, T cells
SMC proliferation/death	Proliferation of SMC	Apoptosis of SMC
Degree of elastin destruction	Slight destruction of elastin in the plaque area	Progressive elastin destruction resulting in media degradation
Consequences	Atherosclerotic plaque rupture causes heart attack, stroke, and peripheral vascular disease	AAA rupture results in bleeding inside the body, frequently fatal

Table 1. Differences between atherosclerosis and AAA. (Peshkova et al., 2015)

1.2.3 Histological features of AAA

The destruction of the lamellar architecture of the aortic media is the predominant histopathological feature of aneurysm disease. As previously described, ECM represents the dynamic structure that supports the aortic wall, driving its main functions. ECM remodeling is strictly regulated under physiological conditions and abnormal expression and activity of the main ECM remodeling mediators are characteristic of many vascular disorders. Elastin fragmentation represents an early event in AAA formation, consequently causing wall weakening; degradation of fibrillar collagen type I and III occurs in a later phase of the AAA expansion, leading to the loss of aortic tensile strength and causing a decisive step in the aortic rupture. Many experimental data, obtained from human aneurysm tissues, as well as from *in vitro* and *in vivo* animal models, highlighted the MMPs involvement in ECM abnormal degradation, evidenced by an increased expression of these proteolytic enzymes during aneurysm initiation and/or progression, correlating with aneurysm size. MMPs are secreted by vascular and inflammatory cells; MMP over-expression in terms of mRNA, protein, as well as enzymatic activity, is not only associated with an increased production and secretion, but it is also the result of the unbalanced MMPs/TIMPs ratio. MMP-2 and MMP-9 are the most commonly enzymes associated with aneurysm disease. They both degrade elastin and collagen. MMP-2 is constitutively expressed in small aneurysm, suggesting a role in aneurysm initiation and formation, whereas MMP-9 is more prevalent in large diameter aorta, indicating a main participation in aneurysm progression and expansion. In addition, MMP-8 is a type I collagenases and, together with MMP-9, was shown to be increased in ruptured aneurysms. In addition to MMPs, other proteases localize at aneurysm wall: u-PA (urokinasePlasminogen activator) and t-PA (tissue-Plasminogen activator) convert plasminogen into plasmin, which in turns activates MMPs; cathepsin S and K, with elastolytic action, and cathepsin L, which degrade type IV and V collagen, laminin, elastin and proteoglycans. Medial SMCs constitute the main vascular cell population that participate to aortic structure, through the synthesis of ECM components as well as the secretion of ECM remodeling mediators, like MMPs and their tissue inhibitors TIMPs. The importance of SMC behaviour in the aneurysm development was explored by Lopez-Candales et al, underlining an increased SMC apoptosis, marked by an increased expression of p53, which is indicative of cell cycle arrest, in aneurysmal tissues versus non-aneurysmal and atherosclerotic aortic tissues (Lopez-Candales et al., 1997)(Holmes et al., 1996). The elastin degradation is tightly related to the abnormal hemodynamic shear stress that influences aneurysm growth. The SMCs respond to elastin degradation through an increased tropoelastin synthesis; this is confirmed by increased mRNA levels of tropoelastin in aneurysm tissue. This adaptation is not followed by the correct elastin

organization into the mature effective structures, resulting in the aortic wall inability to respond to the increased shear stress.

Moreover, another feature of AAA is the intraluminal thrombus (ILT). In 70–80% of AAA patients, the vessel wall is covered by an intraluminal thrombus which generally does not preclude blood flow and shows little compression throughout the cardiac cycle (O'Leary et al., 2014)(Vorp et al., 1996). While mural thrombosis is frequently observed in aneurysmal disease, the complete vessel occlusion is a comparably rare event associated with a high rate of mortality (Cervantes et al., 1985). In recent studies, more attention has been dedicated to the mechanical properties of the ILT. Indeed, the mechanical role of ILT on the wall stress distribution and the underlying local wall strength is yet unclear.

In addition to the loss of structural integrity, aortic aneurysm is characterized by an intensive transmural inflammation. The presence of the inflammatory infiltrate may be due to elastin fragmentation. In fact, this process releases soluble peptides exerting a chemotactic effect that recruits inflammatory cells, which further secrete proteolytic enzymes, thus amplifying the ECM degradation. Studies on the inflammatory infiltrate invading the AAA wall revealed the presence of aggregating clusters of CD3+ T and CD19+ B lymphocytes, together with macrophages, and localized at the adventitial vasa vasorum (Koch et al., 1990). In addition, an imbalance of the ratio between CD3+ T helper and CD8+ T suppressor cells has been observed, with the predominance of T-helper cells (Koch et al., 1990) (Tang et al., 2005). Inflammatory cells activate a cascade of reactions increasing inflammation environment and exacerbating the proteolysis process. CD3+ T lymphocytes produce IL-4, -5, -8, -10 that attract other inflammatory cells, stimulate the cytokine release by T cells and the neoangiogenesis; in addition, CD3+ stimulate the IFN-γ release, which, in turn, induces MMP expression. The presence of antigen presenting cells in aneurysm wall suggests an antigen driven T cell response as leading cause to aneurysm development. Matrix proteins, such as elastin, collagen, aortic aneurysm antigenic protein-40 (AAAP-40) (Xia et al., 1996) and exogenous agent (i.e. herpes simplex virus, cytomegalovirus) have been proposed as potential antigen capable to induce T cells activation. Interestingly, the molecular mimicry is mechanism of T cells activation, and it is supposed to be involved in aneurysm pathogenesis. The molecular mimicry is based on sharing of common sequences and epitops between a foreign antigen (a microorganism) and self (host) antigen: the immune reaction against a virus or bacteria, through a mechanism of cross reaction (Ozsvath et al., 1996)(Oleszak et al., 2004). As-consequence, the immune response inside the aortic wall is exacerbated, thus complicating the proteolytic process. According to these findings, an

autoimmune origin of AAA has been proposed (Hirose and Tilson, 2001). Macrophages actively participate to parietal remodeling and inflammation: immunohistochemical and *in-situ* hybridization data revealed an intensive MMP-9 expression by aneurysm-infiltrating macrophages (Thomson et al., 1995); later works demonstrated the macrophage-mediated release of cytokines, such as Il-1β, TNF-α, IL-8, thus stimulating B-cell and cytotoxic T-cell differentiation, cytokine and protease synthesis. Neo-angiogenesis consists in the formation of new blood vessels from pre-existing ones and for this purpose it requires the ECM degradation by proteolytic enzymes to promote the ECs migration from mature vessels; these steps are driven by specific growth factors, such as VEGF, PDGF, EGF, TNF-α. The occurrence of neovascularisation in AAA wall has been demonstrated by Thomson et al (1995), evidenced by an increased number of neo-vessels in aneurysmal tissues in comparison to normal aortic specimens; moreover, the degree of neovascularisation was shown to be correlated with the inflammatory infiltrate amount (Holmes et al., 1995). These findings, together with immunolocalization studies showing MMPs expression in correspondence of neo-vessels (Herron et al., 1991), support a crucial role for the medial neo-vessels formation to the aneurysm pathogenesis. Moreover, increased neovascularisation and expression of angiogenic cytokines were detected at the site of rupture, indicating an involvement of the angiogenic response into aneurysm rupture.

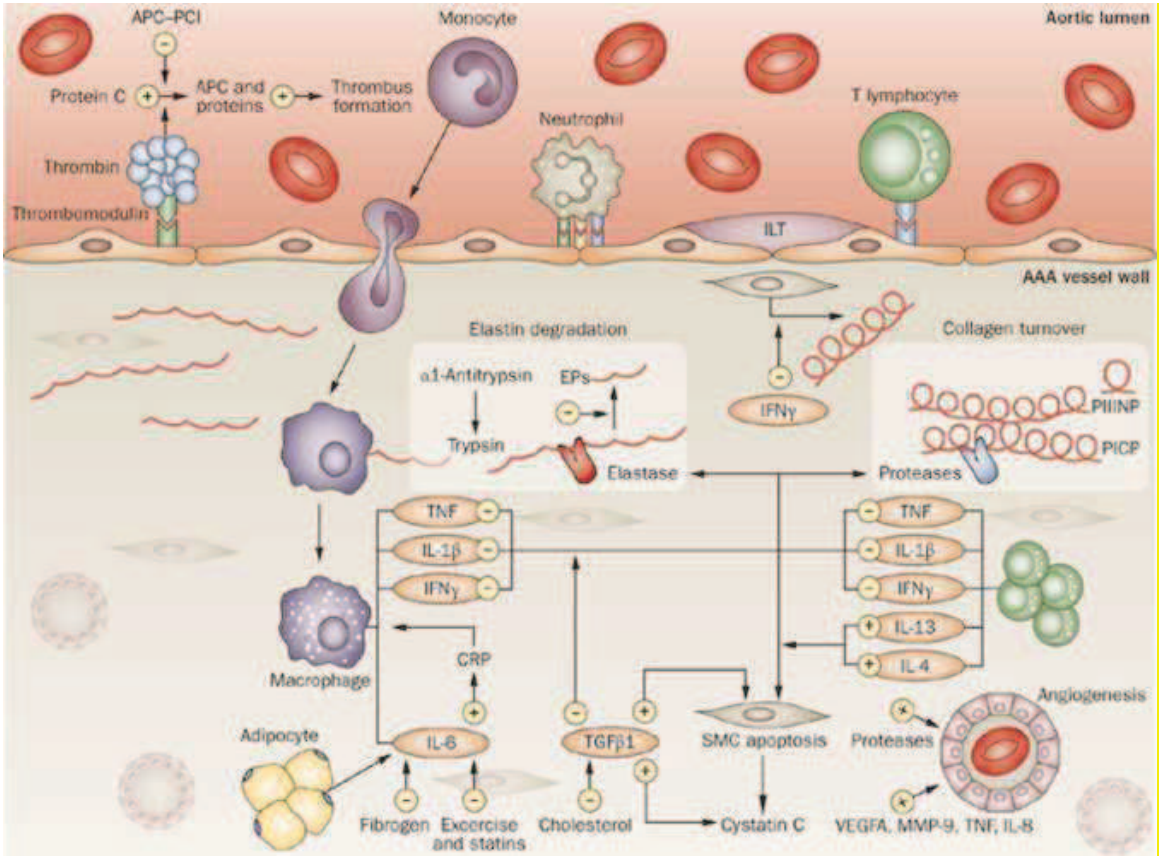


Figure 13. Schematic diagram of the mechanisms involved in abdominal aortic aneurysm, which primarily affect two main processes: inflammation and extracellular matrix turnover (F. a M. V. I. Hellenthal et al., 2009).

1.3 The MMP family

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). MMPs belong to the family of proteolytic enzymes that degrade several components of ECM. MMPs generally consist of a prodomain, a catalytic domain, a hinge region, and a hemopexin domain. The cysteine switch motif PRCGXPD in the propeptide that maintains MMPs in their zymogen form (proMMP), and the zinc-binding motif HEXGHXXGXXH in the catalytic domain is used to distinguish the proteinases family. They are either secreted from the cell or anchored to the plasma membrane. On the basis of substrate specificity, sequence similarity, and domain organization, vertebrate MMPs can be divided into six groups (Figure 14). The key feature of collagenases (MMP-1, MMP-8, MMP-13 and MMP-18) is the ability to cleave interstitial collagens I, II, and III at a specific site. MMP-2 and MMP-9 also belong to collagenases group. They digest denaturated collagens, gelatin. These enzymes have three repeats of type II fibronectin domain inserted in the catalytic domain, which bind gelatin, collagen and laminin.

Stromelysin's group are: stromelysin 1 (MMP-3) and stromelysin 2 (MMP-10) with similar substrate specificities, while MMP-3 has a proteolytic efficiency higher than MMP-10. MMP-11 is called stromelysin 3, but it is usually grouped with "other MMPs" because the sequence and substrate specificity diverge from those of MMP-3.

Matrilysins are characterized by the lack of hemopexin domain. Matrilysin 1 (MMP-7) and matrilysin 2 (MMP-26) belong to this group. MMP-7 processes cell surface molecules such as pro-α- defensin, Fas-ligand, pro-tumor necrosis factor (TNF-α), and E-cadherin, while MMP-26 digests several ECM components. There are six membrane-type MMPs (MT-MMPs): four are type I transmembrane proteins (MMP-14, MMP-15, MMP-16 and MMP-24) and two are glycosylphosphatidylinositol (GPI) anchored proteins (MMP-17 and MMP-25). Seven MMPs are not classified in the above categories. MMP-12 (mainly expressed by macrophages) digests elastin and other ECM molecules. MMP-19, MMP-20, MMP-22, MMP-23 and MMP-28 are part of this group.

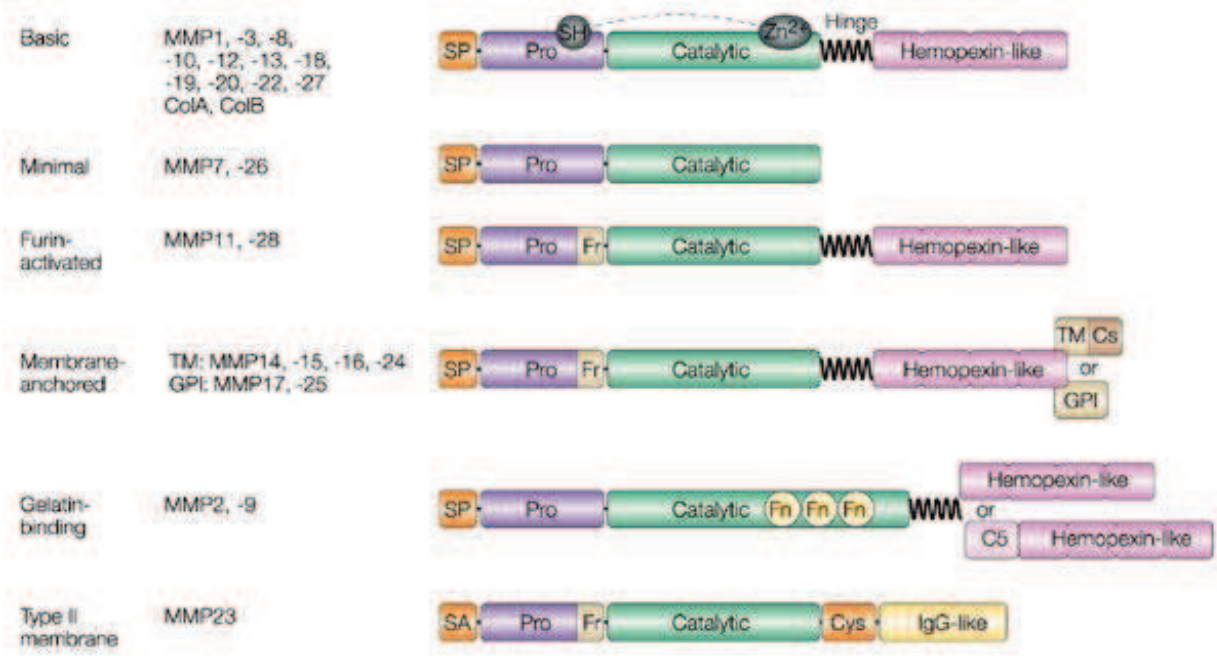


Figure 14. Classification of MMP family. The main features of matrix metalloproteinases (MMPs) are illustrated, showing the minimal domain structures. One clear division is between MMPs that are secreted and those that are anchored to the cell surface by an intrinsic motif namely, a transmembrane (TM) domain (MMP-14, -15, -16 and -24), a glycosylphosphatidylinositol (GPI) anchor (MMP-17 and MMP-25) or an amino (N)-terminal signal anchor (SA) (MMP-23). Nine MMPs, including all of the membrane-anchored enzymes, have a furin-recognition domain. C5, type-V-collagen-like domain; Col; collagenase-like protein; Cs, cytosolic; Cys, cysteine array; Fn, fibronectin repeat; Fr, furin-cleavage site; Pro, pro-domain; SH, thiol group; SP, signal peptide; Zn, zinc. (Parks et al., 2004)

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 amplify their effectiveness (Nagase et al., 2006) (Visse and Nagase, 2003). One of the features of MMP is that many of those genes are "inducible" by different effectors such as growth factors, cytokines, chemical agents, physical stress, and oncogenic cellular transformation. Moreover, MMPs are regulated at different levels: gene expression, localization (that is, the pericellular accumulation of enzyme), pro-enzyme (or zymogen) activation and enzyme inactivation (Ra and Parks, 2007). 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 MMPs activity increasing and, thus, to pathological changes in the vessel wall structure associated with vascular disease (Raffetto and Khalil, 2008)

1.3.1 Focus on human MMP-9

Human MMP-9 consists of an NH2- terminal pro-domain, a catalytic domain, a linker domain, and a COOH-terminal hemopexin-like domain that combines to form a 92 kDa pro and 88 kDa active enzyme. (Papazafiropoulou and Tentolouris, 2009) The catalytic domain of MMP-9 contains two zinc ions, five calcium ions, and three repeats homologous to the type II module of fibronectin. The catalytic zinc ion is essential for the proteolytic activity. MMP-9 has a unique domain termed "fibronectin-like domain", which consists of three repeats of fibronectin type II of about 58 amino acids. This domain is heavily O-glycosylated and contains elongated linker between catalytic and hemopexin-like domain (Opdenakker et al., 2001). The fibronectin-like domain is essential for binding to denatured collagen or gelatin (O'Farrell and Pourmotabbed, 1998). Hemopexin-like domain shares a sequence similarity to plasma hemopexin and it is present in MMP-9. In pro-MMP-9, the hemopexin-like domain forms a tight complex with TIMP-1 and TIMP-3 though their COOH-terminal domains (Nagase et al., 2006), Pro-MMP-9 is complexed with TIMP-1 in the Golgi apparatus of the cell before secretion (Roderfeld et al., 2007). TIMP-1 is bound to the pro-MMP-9 via COOH-terminal domain, leaving the NH2 terminus capable of inhibiting other MMPs.

The expression of MMP-9 is regulated on multiple levels: transcription, post-translation (also involving non-proteolytic activation); local translation (Dziembowska et al., 2012); sequestration on the cell membrane (e.g. binding to cell adhesion molecules, such as hyaluronian receptor CD44 (Bourguignon et al., 1998), integrins (Wang et al., 2003), lipoprotein receptor-

related protein-1 (LRP-1), and megalin/LRP-2 (Van den Steen et al., 2006)); internalization (Hahn-Dantona et al., 2001); and delayed activation that involves cleavage of the propeptide and co-secretion with TIMP-1, its endogenous inhibitor (Sbai et al., 2010).

At the transcriptional level, MMP-9 is positively regulated by multiple factors, including E-26 (Ets) transcription factors, NF-kB, polyomavirys enhancer A-binding proteins-3 (PEA3), activator protein-1 (AP-1), specificity protein 1 (Sp-1), and serum amyloid A-activating factor (SAF)-1 (16). Ets are a family of transcription factors associated with a variety of biological functions including cellular differentiation, cell migration, proliferation, apoptosis and angiogenesis. NF-kB is capable of binding to kB DNA on the promoters or enhancers of genes to regulate expression. Bond et al. reported an increase in MMP-9 levels in vascular smooth muscle cells via NF-kB mechanisms (Bond et al., 2001a). Inhibition of transcription factor NF-κB reduces MMP-9 production in vascular smooth muscle cells and macrophages (Bond et al., 2001) (Grimm et al., 2006). Reactive oxygen species can activate MMP-9, both directly and indirectly, by activating transcription factors such as NF-κB (Okamoto et al., 2001). Ang II has direct and indirect effects on MMP-9 expression. In ventricular myocytes, Ang II directly stimulates NF-κB to induce MMP-9 expression (Rouet-Benzineb et al., 2000). Ang II activates epidermal growth factor receptor and the mitogen-activated protein kinase pathway to induce MMP-9 expression (Shah and Catt, 2003). Aldosterone, which is produced locally in the myocardium, triggers MMP-9 production through NF-κB signaling (Li et al., 2000). PEA3 and AP-1 binding sites are present in the MMP-9 promoter (Wu et al., 2009). AP-1 has two binding sites on MMP-9, and the activation of MMP-9 is preceded by a rapid transient increase in AP-1 protein levels (Woessner, 1991). Thrombospondins stimulate production of MMP-9 by activating AP-1 (Greenwood et al., 1998). Donnini and colleagues showed that fragments of thrombospondin promote MMP-9 production in bovine capillary endothelial cells (Donnini et al., 2004). In order to increase transcription, Sp-1 binds to the MMP-9 promoter to induce transcription. Sp-1 undergoes several post-transcriptional modifications such as phosphorylation and glycosylation (Murthy et al., 2012) (Taheri and Bazan, 2007). Conversely, inhibition of Sp-1 leads to decreased MMP-9 expression (Murthy et al., 2012). In addition, the transcription factor described above cross-interact to regulate MMP-9 expression. In fact in vascular smooth muscle cells, upregulation of MMP-9 is mainly attributed to expression and activation of NF-κB and AP-1 transcription factors (Valen et al., 2001). AP-1 alone, however, is not sufficient for maximal MMP-9 transcription, and the cooperation of either NF-κB or Sp-1 binding proteins upstream of the AP-1 site is required for full MMP-9 transcription induction (Benbow and Brinckerhoff, 1997). SAF-1 is an inflammatory responsive transcription factor that induces MMP-9 transcription via cooperation with AP-1 (Ray

et al., 2005). In the MMP-9 promoter region, SAF-1 is located in close proximity to AP-1 elements. Mutation of either SAF-1 or AP-1 greatly affects the induction of the MMP-9 promoter and reduces the ability of SAF-1 and AP-1 to activate transcription (Ray et al., 2005). Among the cytokines capable of regulating MMP-9 expression, an important role is assigned to TNF-α. Alexander and Acott showed that TNF-α triggers the production of MMP-9 through the protein kinase C signal-transduction pathway (Alexander and Acott, 2001). Lau et al. showed TNF-α upregulated MMP-9 expression in coronary arteries (Lau et al., 2009) (Lau et al., 2008). Heat shock protein 60 has been shown to stimulate TNF-α followed by MMP-9 production in macrophages (Kol et al., 1998). In rat embryonic cardiomyoblast cell line H9c2, the NF-κB II binding site within the promoter region of MMP-9 (−626/−617) plays a key role in upregulation of MMP-9 expression by TNF-α induction (Wu et al., 2013). Among other cytokines capable of inducing MMP-9, IL-1β was shown to increase NF-κB and AP-1 in rat myocytes (Long, 2001). MMP-9 expression has been also reported in cardiomyocytes (He et al., 2011). Classical MMP-9 activation includes disruption of the interaction between the zinc molecule in the catalytic domain and the cysteine switch in the pro-domain. This structural modification leads to the cleavage of the pro-form and production of active enzyme. MMP-9 is activated by other MMPs, including MMP-2, -3, -13, -17, and -26 (Fridman et al., 1995)(Knäuper et al., 1997)(Ogata et al., 1992) (Uria and Lopez-Otin, 2000). For example, the activation of pro-MMP-3 by plasmin, which is generated from plasminogen by uPA bound to the uPA receptor on the plasma membrane, leads to activation of pro-MMP-9 (Ramos-DeSimone et al., 1999). Proteolytic enzymes, such as plasmin, urokinase-type plasminogen activator, and tissue-type plasminogen activator, are capable of cleaving the pro-domain to activate MMP-9 (Pepper, 2001). Another example of indirect pro-MMP-9 activation is through the initial activation of MMP-2 and -13 on the cell surface by membrane type-1-MMP (Fridman et al., 1995). Several serine proteases are capable of pro-MMP-9 activation. Tissue-associated chymotrypsin-like proteinase activates pro-MMP-9 in skin tissues from chronic unhealed wounds (Han et al., 2002). Pancreatic trypsin-2 from human carcinoma was an effective pro-MMP-9 activator (Sorsa et al., 1997). Post-translational modifications of MMP-9 are another potent mechanism of increasing extracellular MMP-9 activity. These include *S*-nitrosylation and *N*-glycosylation. MMP-9 has one *S*-nitrosylation site at cysteine and two *N*-glycosylation sites at asparagines in positions 38 and 120 (Kotra et al., 2002) (Martínez-Ruiz and Lamas, 2004). Although MMP-9 is synthesized and secreted in a pro-form, there is evidence that MMP-9 may also be activated intracellularly. Pereira and colleagues reported that activated MMP-9 accumulates in cells undergoing apoptosis, although this does not rule out the possibility that MMP-9 had been activated extracellularly and taken back up (Pereira et al., 2005). Tissue inhibitor

of metalloproteinase (TIMP)-1-free MMP-9 has been shown to accumulate in microvascular endothelial cells in endothelial vesicles after phorbol myristate acetate stimulation (Nguyen et al., 1998). Future studies are warranted to determine whether MMP-9 can be activated intracellularly. Inhibition of MMP-9 is performed by TIMPs binding to the zymogen forms of the enzyme (Goldberg et al., 1992). All TIMPs are known to interact with MMP-9 and inhibit its activity (Brew and Nagase, 2010). TIMP-1 binds to pro-MMP-9, in addition to inhibiting its active form (Roderfeld et al., 2007). In circulation, α_2 macroglobulin inhibits MMP-9 to prevent systemic MMP-9 activation.

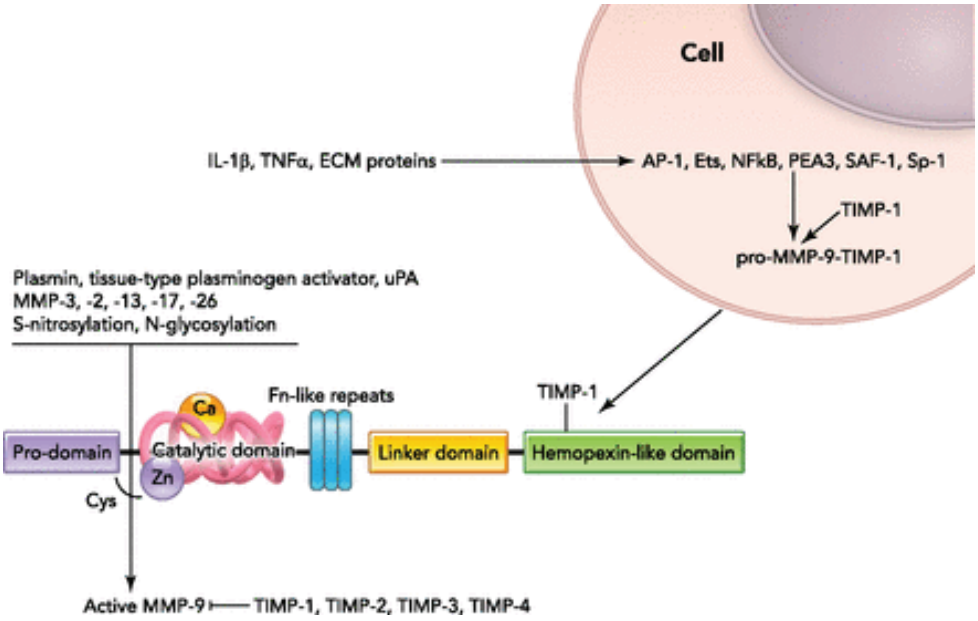


Figure 15. MMP-9 structure and factors regulation MMP-9 transcription and translation. (Yabluchanskiy et al., 2013)

Finally, several ECM proteins are proteolytically processed by MMP-9, including elastin, collagen, fibronectin, and laminin. ECM fragments are known to express bioactive properties vascular remodeling (Trial et al., 2004). Laminin is a well-known MMP-9 substrate, and its levels negatively correlate with increased levels of MMP-9 (Horstmann et al., 2003). MMP-9 is known to process a number of inflammatory chemokines through proteolysis. MMP-9 processes CXCL5 at the NH2 terminus and increases twofold its chemotactic activity (Van Den Steen et al., 2003). MMP-9 processes several cytokines, including TNF-α, IL-1β, and TGF-β. MMP-9 was shown to release active TNF-α from the cell surface via proteolysis, which results in the production of a biologically active mature form (Gearing et al., 1994). TGF-β is an anti-inflammatory cytokine and it is released in the extracellular space in a latent form. Its maturation is associated with several mechanisms, including proteolysis. MMP-9 can cleave the latent pro-form of TGF-β to its active state (Yu and Stamenkovic, 2000).

1.4 Molecular pathway included in abdominal aortic aneurysm

Several molecular pathways are involved in the pathogenesis of AAA. Mediators of aortic damage include angiotensin II, leukotriene-LT4, prostaglandin- PGE2, interleukins, tumor necrosis factor, tissue plasminogen activator, c-Jun N-terminal Kinase, NF-kB, Rho kinases and osteoprotegerin. They activate matrix metalloproteinase, serine proteases and cysteine proteases. The result is the degradation of aortic wall proteins, extracellular matrix and the apoptosis of vascular smooth muscle cells. (Nanda et al., 2009)

- Cytokines: Tumor Necrosis Factor-α (TNF-α)

TNF-α is a 17 kDa nonglycosilated soluble protein, derived by the cleavage of the 32 kDa transmembrane precursor mediated by the TNF-α converting enzyme. TNF-α is a critical member of immune system and produces pro-inflammatory alteration in cells implicated in AAA. TNF-α activates a large spectrum of biological activities, among which the regulation of cell growth, differentiation, programmed cell-death and it also drives the inflammatory cascade events both under acute and chronic conditions TNF-α could have a central role in the aortic dilatation process by causing smooth muscle cell death and inducing the release of proteases that may weaken the aortic matrix. (Newman et al., 1994) Many investigations have demonstrated increased circulating and tissue levels of TNF-α in AAA patients particularly in small versus large aneurysm (Juvonen et al., 1997) (Hamano et al., 2003)(Satoh et al., 2004). These observations suggested a role for TNF-α in the early stage of aneurysm pathogenesis; this association was further confirmed by studies on animal models, showing that the inhibition of TNF-α protein could prevent aortic dilatation (Hingorani et al., 1998). Macrophages and lymphocytes are the main source of TNF-α, which exacerbates the inflammatory response mediating the release of adhesion molecules, such as VCAM-1 and ICAM-1, the lymphocyte proliferation and also stimulating the MMP secretion from both inflammatory and resident vascular cells, including SMC (Sarén et al., 1996)(Cohen et al., 2006).

- c-Jun N-terminal Kinase

c-Jun N-terminal Kinase (JNK) is highly activated in AAA (Yoshimura et al., 2005). JNK activity is crucial for secretion of MMP-9 and may participate in the proinflammatory cascade. (Bagowski and Ferrell, 2001) Consequently, inhibition of JNK resulted in the marked suppression of MMP activities, cellular infiltration and AAA development in mice. (Yoshimura et al., 2005) JNK activation has a pivotal role in ECM metabolism, its suppress genes encoding ECM proteins and biosynthetic enzymes, such as lox. Moreover, JNK activates numerous genes in the VSMC. Among JNK-induced genes, IL-1α, tropomyosin receptor kinase-C (TrkC), inducible nitric oxide

synthase, MMP-9 and lipocalin-2, are positive regulators of MMP-9 activity. NF-kB synergistically regulates the expression of MMP-9 with AP-1(Sato and Seiki, 1993), which is the major target of JNK pathway. NF-kB and the JNK pathway, however, may not always work synergistically, because NF-kB antagonizes the JNK pathway in TNF-alpha–induced apoptosis. (Papa et al., 2004)

- Oxidative stress

The existence of oxygen and nitrogen reactive species (ROS, RNS) in the aneurysm wall has been widely demonstrated. Oxidative stress includes a series of reactions which are mediated by the release of ROS and RNS by different cell types, inducing cell and tissue damage. These conditions depend on the increased ROS and RNS production, and/or the impaired activity of anti-oxidant systems, such as superoxide dismutases (McCormick et al., 2007). First evidences of ROS and RNS involvement in AAA pathogenesis derive from Dubick et al, that demonstrated a reduced activity of superoxide dismutase in aneurysm tissues, versus normal aorta (Dubick et al., 1987). A comparative study between the aneurysm and the adjacent non-aneurysmal portion of patients undergoing surgical repair revealed a marked oxidative stress in the aneurysm segment evidenced by the increased expression of superoxide and NADPH oxidase, in comparison to the adjacent unaffected neighbours segments; moreover, the O_2^- increase was associated with inflammation, SMC depletion and elastin degradation (Miller et al., 2002). Cells from the inflammatory infiltrate constitute the main source of oxidative stress, especially macrophages generating large amounts of superoxide (O_2^-) and hydrogenperoxide (H_2O_2), thus further contributing to the inflammatory condition and the progression of the disease. Another source of reactive oxygen species is represented by vascular cells, including ECs, SMCs and adventitial fibroblasts. NADPH oxidase seems to play a key role in generating ROS and its activity may be influenced by several and different pathways, including cytokines released by inflammatory cells, lipid mediators, such as leukotrienes, oxidized LDL and growth factors. In addition, the mechanical stretch also exerts a decisive role, since it can stimulate SMC production of ROS through the activity of NADPH oxidase (McCormick et al., 2007). Oxidative stress can activate many biological responses promoting the aortic wall remodeling and dilatation, including osteopontin upregulation by ECs; activation of MMP and vSMC apoptosis.

1.5 Future perspective: Epigenetic in AAA pathogenesis

AAA is a complex disease caused by the interaction of environmental risk factors and genetics. Several mechanisms influence gene expression. The process of controlling gene expression through these method is known as epigenetics and includes RNA associated silencing, histone modifications and DNA methylation. (Jones, 2012)

- *Role of DNA methylation in AAA*

DNA methylation is a process in which a methyl group is added to a region where a cytosine nucleotide, located next to a guanine nucleotide, linked by a phosphate (CpG). A cluster of CpGs is called CpG island (CpGI). CpGIs are methylated by a group of enzymes called DNA methyltransferases. Insertion of methyl groups at CpGIs was thought to block the binding of transcription factors to promoters and therefore result in repressed gene expression.

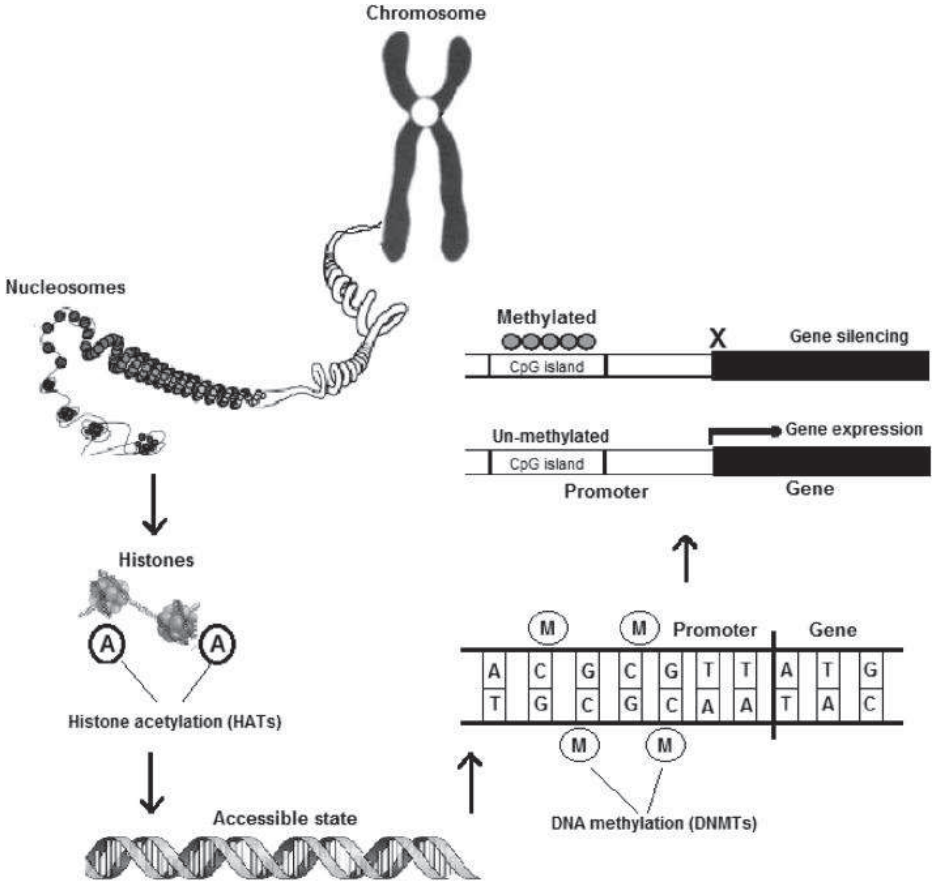


Figure 16. Overview of epigenetic processes. CpG methylation is characterised by the addition of a methyl group (CH_3) to the 5th carbon of a cytosine base that is 5′ to a guanine. DNA methyltransferase (DNMT) enzymes are responsible for this. CpG islands (dense regions of CpG dinucleotides within gene promoter regions) can have abnormally high (hyper) or low (hypo) levels of DNA methylation in many disease processes. DNA methylation of gene promoter regions inhibits gene transcription due to interference of the transcriptional binding complex essential for the recruitment of RNA polymerases. (Toghill et al., 2015)

Changes to DNA methylation in genes controlling the regulatory cycles of ECM proteolysis can specifically influence phenotypic alteration of the structure and function of the ECM. Cigarette smoking (a risk factor for AAA development) is a powerful environmental modifier of DNA methylation and it is a potential mechanism by which tobacco can affect gene expression. Ryer et al. using isolated human mononuclear blood cells and controlling for smoking status, showed significant differences in DNA methylation at specific CpG island that mapped to two genes: CNN2 and SERPINB9 (Ryer et al., 2015). The first gene, CNN2, which is also known as h2-calponin or calponin, is an actin-binding protein implicated in cytoskeletal organization and vascular development. The second gene, SERPINB9 belongs to a family of serine protease inhibitors present in the cytoplasm of lymphocytes. SERPINB9 has been shown to inhibit apoptosis of human vSMCs, again a key process in AAA pathogenesis. In conclusion, DNA methylation has a potential role in the pathogenesis of AAA. (Toghill et al., 2015)

- *Role of Micro-RNA in AAA*

Over the past decades, also micro-RNAs (miRs) have emerged as major post-transcriptional regulators of gene expression and it has been shown to play key roles in vascular biology and cardiovascular diseases. (Wei et al., 2013)(Mcmanus and Freedman, 2015) (Caputo et al., 2015) Intracellular miRs mainly regulate gene expression through binding to their target messenger RNA, inhibiting their translation, or inducing their degradation. Briefly, miRNA is initially transcribed as primary miRNA (pri-miRNA), then processed into precursor miRNA (pre-miRNA) by a microprocessor complex composed of Drosha and DiGeorge syndrome critical region 8 (DGCR8), pre-miRNA is then transported from the nucleus to the cytoplasm by exportin 5, in presence of the Ran-GTP cofactor, and further processed into its mature form by Dicer. The miRNA is then recruited to the RNA-induced silencing complex (RISC) and it regulates the output of protein-coding genes through diverse mechanisms. The interaction of miRNAs with the 3′ untranslated region (3′ UTR) of protein-coding genes is considered as the main mechanism, which usually leads to a decrease in protein output either by mRNA degradation or by translational repression. Recent studies have also suggested that miRNAs can interact with the 5′ UTR of protein-coding genes via complementarity and cause translational repression (Lytle et al., 2007) or activation of the targeted proteins (Ørom et al., 2008). The importance of miRs for vascular homeostasis becomes clear with the generation of mice with endothelial-specific depletion of Dicer that displayed postnatal angiogenesis defect. (Suárez et al., 2008) Many microRNAs have been identified to control crucial biological processes in cardiovascular system.

(Bonauer et al., 2010) For example, microRNA 143 and -145 were shown to control vSMC phenotype (Cordes et al., 2009) and microRNA-126 EC function. (Fish et al., 2008) Several clinical studies have been set up to identify microRNA as new biomarkers for AAA. Indeed, miRs were found up-regulated in aneurysmal tissue others down-regulated. Keiwa et al demonstrated that miRNA expression profile in human AAA wall tissue was quite different from the profile obtained from normal aortic wall tissue, including endothelial (let-7f and miR-20a, -21, -27, -92a, -126, -221, and -222) and inflammatory (miR-124a, -146a, -155, and -223) microRNAs. They found that vessel wall–related and inflammatory cell–derived miRNAs were significantly upregulated in aortic aneurysm wall tissue. These miRNA expression changes were closely correlated with inflammatory cytokines, including MCP-1, TNF-α, and TGF-β, which are responsible for AAA pathophysiology. Moreover, they analyzed the circulating miRNA expression profile of patients with AAA. Interestingly, miRNAs that were upregulated in human AAA wall tissue were significantly downregulated in plasma from patients with AAA.

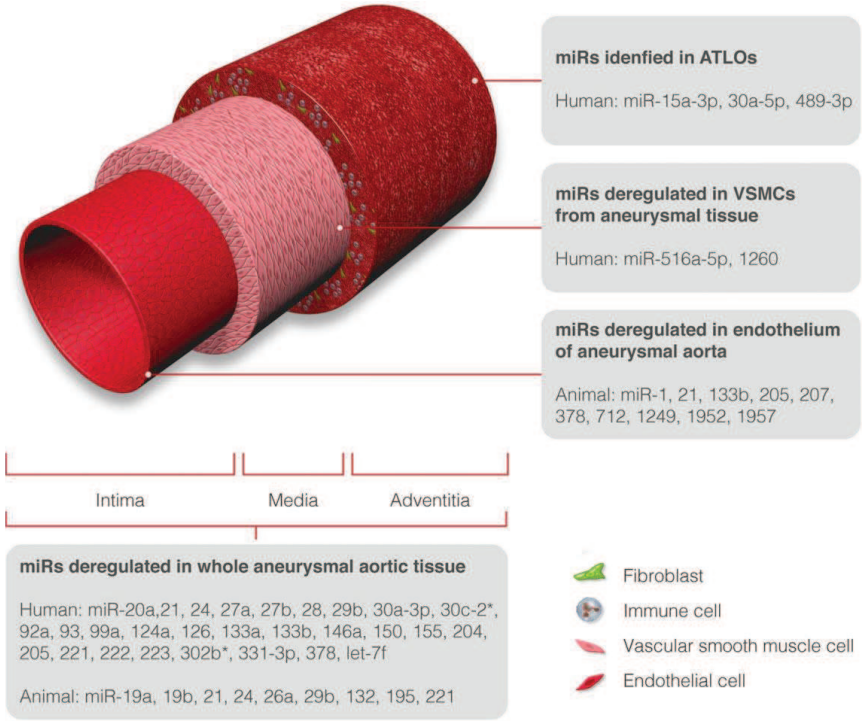


Figure 17. Main miRs identified in aortic tissues during AAA. mRs are differentially expressed during AAA, whether analysed in whole aortic tissue, or in distinct aortic layers such as the endothelium or the VSMC layer. mRs have also been identified in artery tertiary lymphoid organs that form in the adventitial layer during AAA. AAA, abdominal aortic aneurysm; ATLOs, artery tertiary lymphoid organs; VSMC, vascular smooth muscle cells; miRs, micro-RNAs. (Raffort et al., 2016)

Recent studies showed that microRNA-29 plays a key role in AAA formation (Boon et al., 2011; Merk et al., 2012). These studies reported that inhibition of microRNA-29 reduces AAA in different murine models. In particular, microRNA-29 regulates the expression levels of

multiple targets with a function in ECM (collage, elastin and fibrillin), and the inhibition of microRNA-29 improves the structural integrity of vessel wall. Interestingly, inhibition of microRNA-29a, a member of microRNA-29 family, can also increase tropoelastin expression in venous vSMCs to increase elastin deposition in bioengineered vessels. (Rothuizen et al., 2016)

In conclusion, clinical studies revealed concomitant deregulation of some miRs and their putative targets, some of them being key players in mechanisms involved in AAA formation, including inflammation, fibrosis, or ECM remodeling, suggesting potentially causal roles of miRs in the pathophysiology of human AAA.

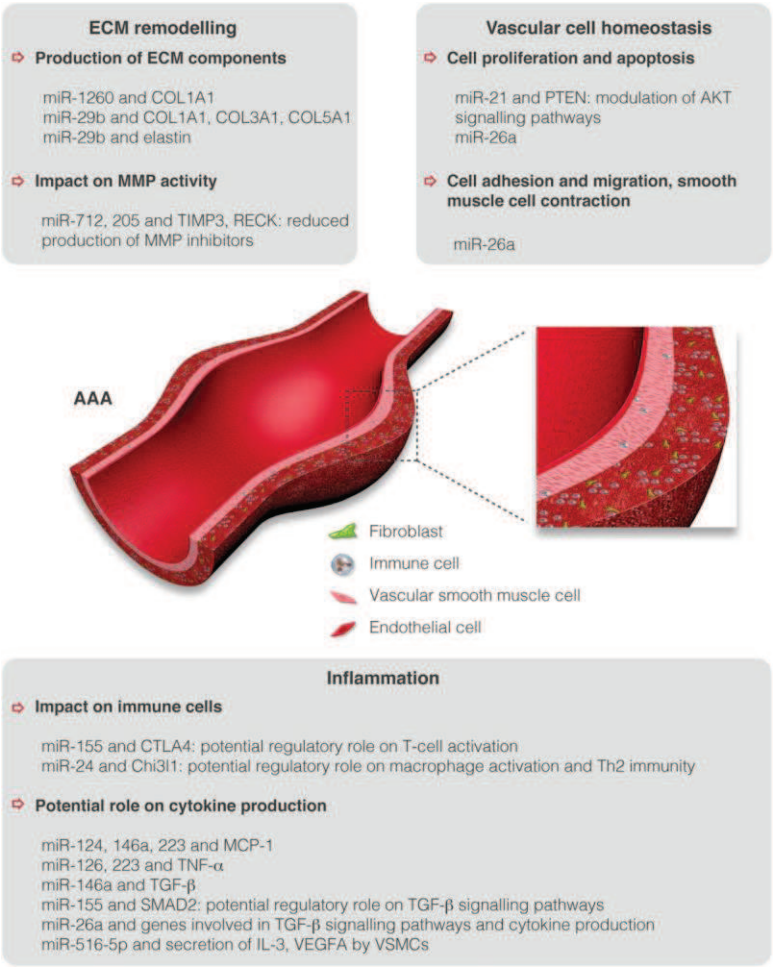


Figure 18. Implication of miRs in pathogenic pathways of AAA revealed by concomitant variation of miRs and their predicted targets. miRs are involved in ECM remodeling through their impact on the production of ECM components such as elastin or collagen or via a regulatory role on MMP activity. They regulate vascular cell homeostasis by targeting key molecules involved in cell proliferation, apoptosis, adhesion, and migration. They are also involved in the inflammatory process that characterizes AAA through their modulatory effects on immune cells and cytokine production. AAA, abdominal aortic aneurysm; Chi3l1, chitinase 3-like 1; COL1A1, collagen type 1, alpha 1; COL3A1, collagen type 3, alpha 1; COL5A1, collagen type 5, alpha 1; CTLA4, cytotoxic T-lymphocyte-associated protein; ECM, extracellular matrix; IL-3, interleukin-3; MCP-1, monocyte chemoattractant protein-1; MMP, metalloproteinase; PTEN, phosphatase and tensin homologue; RECK, reversion-inducing cysteine-rich protein with kazal motifs; TGF-b, transforming growth factor beta; Th2, T-helper lymphocyte, type-2; TIMP3, tissue inhibitor of metalloproteinase-3; TNF-a, tumour necrosis factor alpha; VEGFA, vascular endothelial growth factor A; miRs, micro-RNAs; VSMC, vascular smooth muscle cell. (Raffort et al., 2016)

2. Aim of the project

The project is focused on the pathogenesis of aneurysms, in particular abdominal aortic aneurysm (AAA). This pathology is mainly related to the loosening of the biomechanical properties of the vessel wall. AAA is a multifactorial degenerative disease associated with inflammatory infiltrate, degradation of extracellular matrix (ECM) and apoptosis and phenotype switching of vascular smooth muscle cells (vSMC).

The aim of this project was the study of the involvement of endothelial cells and endothelial MMP-9 in AAA progression. At this regard, the first part of the thesis is focused on histological analysis on AAA specimens. Moreover, in order to better understand the role of ECs and the role of endothelial MMP-9 in AAA progression, neo-angiogenesis and cell function in MMP-9-silenced ECs have been characterized. MMP-9 silencing was obtained through shRNA delivered by lentiviral vector. The role of matrix-metalloproteinase 9 (MMP-9) as a potential trigger of the inflammatory during the interaction between endothelial cells and vascular smooth muscle cells, and between endothelial cells and macrophages was characterized. This project investigates MMP-9 as a novel therapeutic target to restore vascular ECM in aneurysm context.

3. Material and Methods

3.1 Study of protocol and sample collection

Aneurysm tissues of the abdominal aortic segment were provided by the Vascular Surgery Unit, University Hospital Maggiore, Novara. AAA tissues were collected from 15 patients (89% male, mean age 75±6) subjected to surgical repair; demographical and clinical data were reported on a database (Figure 21A). All samples were collected from donors correctly informed for the use of excessive pathological material for diagnostic and research purpose according to the local institute's regulation and policies.

Control aortic wall tissues derived from 3 healthy donors were provided from the Cardiovascular Tissue Bank, University Hospital S. Orsola-Malpighi, Bologna. Vascular tissues included in our study derive from the arterial segments not available for surgical use. Control aortic segments were selected according to two specific criteria: age between 18 and 55 years, to exclude ageing process; cause of death not associated with cardiovascular pre-existent affections, or brain haemorrhage.

3.2 Immuno-histological analyses

In order to characterize aortic wall and identify typical aneurysm features, AAA tissues were in part reserved for histological exam. Briefly, tissue samples were rinsed in phosphate-buffered saline (PBS) and fixed in 10% formalin for 48h at 4°C. Samples were subjected to several dehydration steps of 60 min incubation in solutions with increasing ethanol content (50%, 70%, 95% and 100%) and finally 1h incubation in xylene. Then, 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, weigert, sirius red, masson's trichrome and alcian blue PAS stainings.

Immunofluorescence and immunohistochemistry were performed on deparaffinized sections. For immunohistochemistry, after antigen retrieval (Sodium citrate buffer 10 mM, pH 6.0) with a pressure cooker, the primary antibodies were bound and detected using the VectaStain Elite Kit.

Anti MMP-9 antibody was used (1:500, ThermoFischer, Italy). Reveal was performed by the use of diaminobenzidine (Vector Labs, US). Hematoxylin was used as counter staining (Sigma, Italy). Rabbit polyclonal anti-human C4d antibody has been used by an automated immunostainer (Ventana, Roche, Italy). All images were acquired using Pannoramic MIDI 3DHISTECH and analyzed with Pannoramic Viewer software (3DHISTECH, Hungary).

3.3 Gelatin zymography for detection of MMP-2 and MMP-9

Human tissues from aneurysm and healthy tissues have been lysed with RIPA buffer (150mM sodium chloride, 1% Triton X100, 0,5% Sodium deoxycholate, 0,1% sodium dodecyl sulfate, 1mM EDTA, 1mM EGTA, 50 mM TRIS pH=8) supplemented with protease inhibitors (all from Sigma Aldrich, Italy). Protein samples and non-reducted conditioned media samples (FBS free conditions) obtained from endothelial cells were resolved by SDS-PAGE gels containing 0.2% gelatin (Sigma Aldrich). Briefly, after electrophoresis, gels were incubated with TRITON X-100 for 3h at room temperature, and then incubated overnight at 37°C in a solution of $CaCl_2$ (1mM) and NaCl (15mM), pH 7.4 Following, gels were fixed and stained with coomassie blue. For objective quantification, ImageJ software has been used.

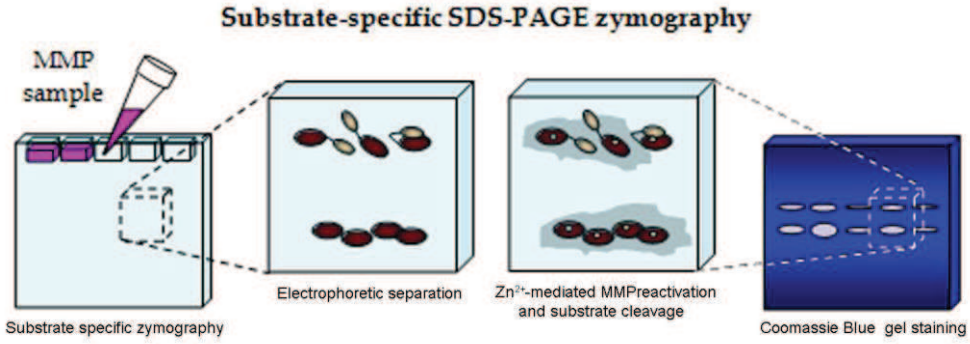


Figure 19. Schematic representation of gelatin zymography

3.4 Cell culture

Human endothelial cells, EA.hy926 (ATCC® CRL-2922™) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) additioned with 10% fetal bovine serum and penicillin and streptomycin (100U/mL) and glutamine (2mM) (all from Euroclone, Italy) at 37°C in humid 5% CO_2 atmosphere. Primary human aortic vSMCs (ATCC® PCS-100-012™) enriched with Vascular Cell Basal Medium (ATCC® PCS-100-030™) in the presence of Vascular Smooth Muscle Cell Growth Kit (ATCC® PCS-100-042™) and Penicillin-Streptomycin-Amphotericin B Solution (ATCC® PCS-999-002™). vSMCs were used up to the 8th passage. U-937 (ATCC® CRL-1593.2™), human monocytes, were used for adhesion assays an endothelial monolayer. U-937 cells were cultured in RPMI-1640 medium additioned with 10% fetal bovine serum and penicillin and streptomycin (100U/mL) and glutamine (2mM).

3.5 Human peripheral blood mononuclear cells (PBMCs) isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque™ Premium (GE Healthcare, Italy) density gradient centrifugation. Diluted peripheral blood (1:2 dilution with PBS) was gently stratified on Ficoll-Histopaque solution and, then, centrifuged at 1500 rpm for 30'. Blood components resulted separated according to their density: plasma, platelets and lymphocytes concentrate above the Ficoll, whereas erythrocytes and polymorphonucleated cells, in virtue of a higher density, concentrate below the Ficoll. The white cell ring was recovered and washes twice with PBS. To promote adhesion of isolated monocytes, peripheral blood mononuclear cells were cultured in serum-free RPMI medium. After 30 min, medium was replaced with RPMI containing 5% fetal bovine serum, and cells were cultured for 12 h. To generate macrophages, cells were cultured in DMEM with 10% fetal bovine serum, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 0.25 mM HEPES (Lonza, Italy), and 10 ng/mL macrophage colony-stimulating factor (ImmunoTools, Germany) for 7 days.

3.6 Cytofluorimetric analysis for macrophages characterization

Cells, detached from culture dishes with 10 mM EDTA, were incubated for 30 min on ice with PE-labeled anti-CD11b and anti-105, FITC-labeled CD14, as suggested by the manufacturer's indications. Cells were analyzed by using a FACScalibur flow cytometer (BD Biosciences, Italy).

3.7 Lentiviral vector transduction

MMP-9 silencing was performed in EA.hy926 cells using lentiviral vector (LV) expressing shRNA targeting the human MMP-9. Two different shRNAs DNA sequences were acquired:
shRNA1, TRCN0000373061 – CCGGGCCGGATACAAACTGGTATTCCTCGAG GAATACCAGTTTGTATCCGGCTTTTTG; and
shRNA5, TRCN0000051438 –
CCGGCCACAACATCACCTATTGGATCTCGAGATCCAATAGGTGATGTTGTGGTTTTTG.
Third-generation LVs were generated as elsewhere described. (Follenzi and Naldini, 2002) 293T cells were cotransfected by calcium phosphate precipitation with the following four plasmids: pMDLg/RRE packaging plasmid; pMD2.VSV-G envelope-coding plasmid; pRSV-Rev and transfer vector plasmids for MMP-9 silencing as reported above. Thirty hours after transfection, culture supernatants containing the viral particles were collected and concentrated by ultracentrifugation. Collected viral particles were used to transduce $5*10^4$ EA.hy926 with two different LVs (shRNA1 and shRNA5). After 3 days of culture, cells were selected with puromycin (2.5 µg/mL) (Sigma, Aldrich, Italy) and used for the experiments.

3.8 Cytokines antibody arrays and immunofluorescent microscopy on EA.hy926 cells

Supernatants collected from control and transduced EA.hy926 (shRNA 5+1) treated with human recombinant TNF-α (50ng/mL) for 24 hours were assayed for a wide panel of cytokines using a Human antibody Array (Panomics, Italy) following the manufacturer's protocol. Briefly, supernatants collected from the different samples were incubated with the array membranes overnight at 4°C. After rinsing, the biotin-labeled detection antibodies have been added to the array membranes and incubated for 2 h. Then, streptavidin-HRP conjugated was added to the membranes and incubated for 1 hour. After rinsing, the array membranes were incubated with the detection solution and then cytokines panel was visualized using enhanced chemiluminescence ECL detection reagents in a chemisensitive visualizer (VersaDoc, BioRad, Italy). For immunofluorescence, anti-MCP-1 (1:50; ab9669, Abcam, UK) and anti-E-selectin (1:50; sc-14011, Santa Cruz Biotechnology, USA) primary antibodies were used following the procedures previously described in details. (Paragraph 3.2)

3.9 Leukocyte-Endothelium Adhesion Assay

U937 cell line -EA.hy926 adhesion assay was performed by Cell Biolabs' CytoSelectTM Leukocyte-endothelium adhesion assay. Wild type and transduced endothelial cells were grown to 80–90 % confluence in gelatin-coated 24-well plates, stimulated with 50 ng/ml TNF-a for 4 h. Then U937 were labelled by the LeukoTrackerTM solution for 1 h at 37 C and washed twice with growth medium. Labelled U937 were incubated together with endothelial cells. After 1 h of incubation, non-adherent cells were removed by PBS rinsing. Adherent monocytes were counted in three separate fields per well by using an inverted fluorescence microscope.

3.10 NF-kB activation

$1*10^4$ ECs were cultured on cover slips in FBS free media overnight. After 10', 30' and 1 hour of TNF-α stimulation, cells were fixed in 4% formalin, and labelled using p65 antibody (F-6, sc-8008, Santa Cruz, Biotechnology, USA). Then, immunofluorescence have been performed. In order to investigate phospho-p65-Ser 536, (sc-33020, Santa Cruz, Biotechnology, USA) and total p65 expression the same timing was used for western blot assay. (Paragraph 3.12)

3.11 Tube formation assay

Growth factor–reduced Matrigel (Corning, USA) enriched with VEGF (200ng/mL), was plated in a 24 multi-well. After Matrigel jellification (37°C for 30 min), EA.hy926 wild type and transduced cells were seeded ($1*10^5$ cells per well) with 300µl of culture media. The cells were

incubated up to 6h at 37°C. Each well was observed and images were acquired using an inverted microscope equipped with digital camera.

3.12 Western blot

Cells were lysed in RIPA buffer. 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 rinsing three times with PBS 0.1% Tween 20, 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).

3.13 Preparation of conditioned media (CM) and silver staining

$2.13*10^4/cm^2$ endothelial cells (wild-type and transduced cells) were grown to subconfluent density in 6-well culture plates in 10% serum-containing medium. After 16h, human recombinant TNF-α (50ng/mL; Sigma Aldrich, Italy) was added to serum-free medium, while wild-type or transduced EA.hy926 cells grown in the absence of TNF-α were used to determine MMP-9 basal expression. After 24h, media were collected as conditioned media (CM) and transferred to human vSMCs ($5.4*10^3$cells/cm^2) for 24h. VSMCs cultured in the absence of CM were used as negative controls. Silver stain plus (161-0449 Bio-Rad, Italy) was performed on vSMC medium collected in the previously described conditions according to the manufacturer's instructions. A Congo Red-elastin (5mg; E0502 Sigma-Aldrich, Italy) (15U of E1250, Sigma-Aldrich, Italy) was used as a positive control of proteolytic digestion.

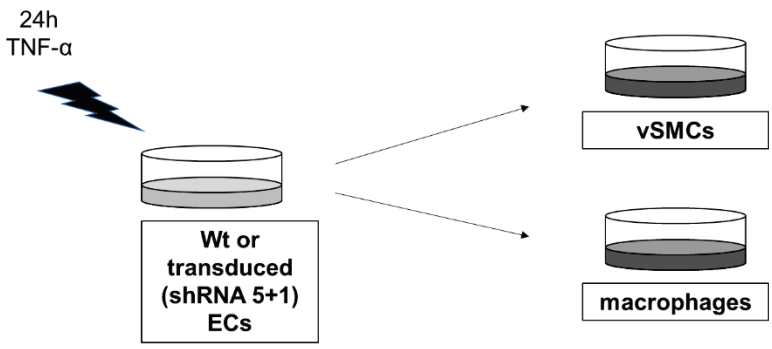


Figure 20. Flowchart of the experimental procedure.

3.14 Statistical analysis

All the experiments were repeated at least three times. All data are expressed as mean values ± standard deviation. The Student t test was performed to confirm the statistical significance of the obtained data. ** indicates $p \leq 0{,}001$ while * indicates $p \leq 0{,}05$.

4. Results

4.1 Study subjects: AAA patiens and control group.

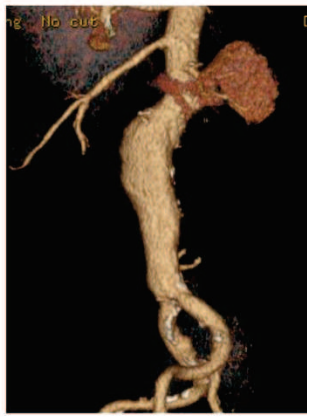
The analysis of the study involved two main groups, represented by I) aortic tissue derived from patients underwent surgical repair for abdominal aortic aneurysm provided by the Vascular Surgery Unit of Ospedale Maggiore in Novara; and II) aortic tissues derived from healthy donor collected from the Cardiovascular Tissue Bank of S. Orsola-Malpighi Hospital (Bologna). Clinic features of AAA patients, such as demographical data, cardiovascular risk and pharmacological treatments (B-blockers, statin, antiplatelet drugs, oral anticoagulant and ACE inhibitors) were recorded prospectively in a database. Figure 21A summarized patient clinical data.

Regarding the control group, we selected aortic tissues deriving from healthy donors of arterial segment, not available for surgical purpose by Cardiovascular Tissue Bank. Selection criteria excluded vascular segments affected by calcifications and included cause of death not associated with cardiovascular affections.

A

Clinical characteristics of AAA Patients	
Years (mean±SD)	75±6
Sex	Male (89%)
DAAA (mean±SD)	5.1 ±0.5 cm
Hypercholesterolemia (%)	44%
Smoking (%)	66%
Hypertension (%)	89%
Ischemic Cardiomyopathy	56%
COPD	44%
CRF (%)	22% stage 2; 22% stage 3
Type 2 Diabetes	0%
B-Blockers	56%
Statins	44% Simvastatin; 11% Atorvastatin
Antiplatelet drugs	22% Clopidogrel; 44% Acetylsalicylic acid
Oral anticoagulants	11% Warfarin
ACE inhibitors	56%

B



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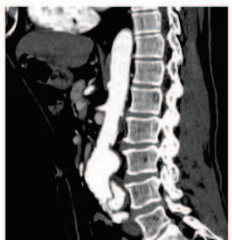

Figure 21. A) Clinical features of AAA patients. DAAA diameter aneurysm, COPD Chronic obstructive pulmonary disease, CRF chronic renal failure. B) 3D image of aneurysm C) Computed Tomography (CT) is the gold standard for the diagnosis of AAA.

4.2 Histological features of aneurysm-affected abdominal aorta

Histological stainings were performed on 5 µm sections of AAA and control groups. In presence of aneurysms, the vessel wall microstructure resulted significantly modified. Distinctive feature of AAA is represented by the complete loss of the vessel wall architecture, so the three wall layers could not be distinguished; this condition is due to the loss of muscular tone and extensive medial degeneration, as observed by histological analyses. In addition, aneurysmal aortae had significantly increased adventitial vasa vasorum densities and in the medial layer compared with controls. AAA is associated with the high degree inflammatory infiltrate: this aspect can be revealed by the presence of many immune cells, including lymphocytes and macrophages as shown in haematoxylin-eosin staining. (Figure 22A) Moreover, AAA is characterized by a loss of vSMCs. In control tissues, the majority of cells in the tunica media are smooth muscle cells represented by elongated nuclei, and in the medial layer cells are strongly positive to alpha-sma antibody. (Figure 22B) Smooth muscle cells and collagen fibres are found between the layers of elastic fibres in the media layer. The subendothelial layer of connective tissue is characterised by a lower density of cells, i.e. fewer nuclei.

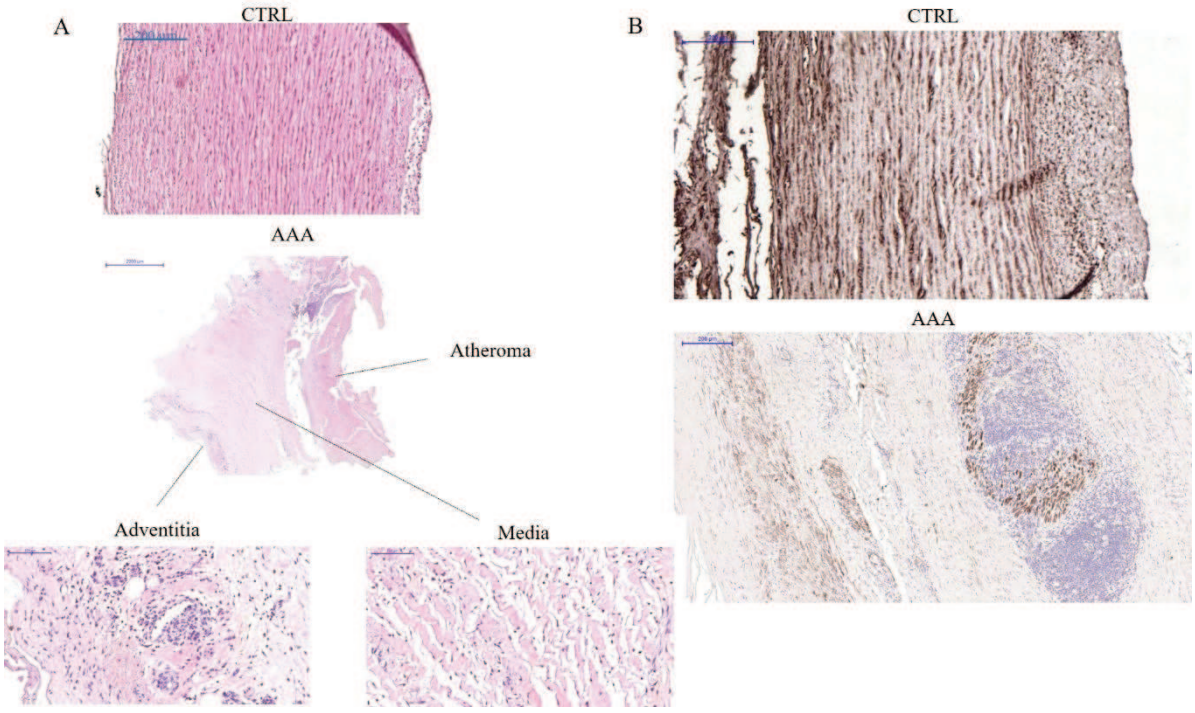

Figure 22. Representative stainings on control and AAA sections. A) Hematoxylin/eosin on CTRL, scale bar 200µm; and hematoxylin/eosin on AAA, 2000µm; adventitia and media layers 100µm. B) Representative immunhistochemistry anti-alpha-sma staining on healthy tissue, scale bar 200µm, and on medial layer of AAA, scale bar 200µm.

In AAA, Masson's trichrome and Sirius Red showed thickening of collagen fibers. (Figure 23C and 23F) Elastic fibers in the medial layer were irregularly arranged and frequently fragmented, instead, in control aortae, elastic fibers had regular black linear structures, as shown by Weigert staining. (Figure 23I). In aneurysms, alcian-blue PAS staining showed cystic medial necrosis (CMN). (arrows in Figure 23L) CMN is a disorder of large arteries, such as aorta, characterized by an accumulation of basophilic ground substance in the media with cyst-like lesions.

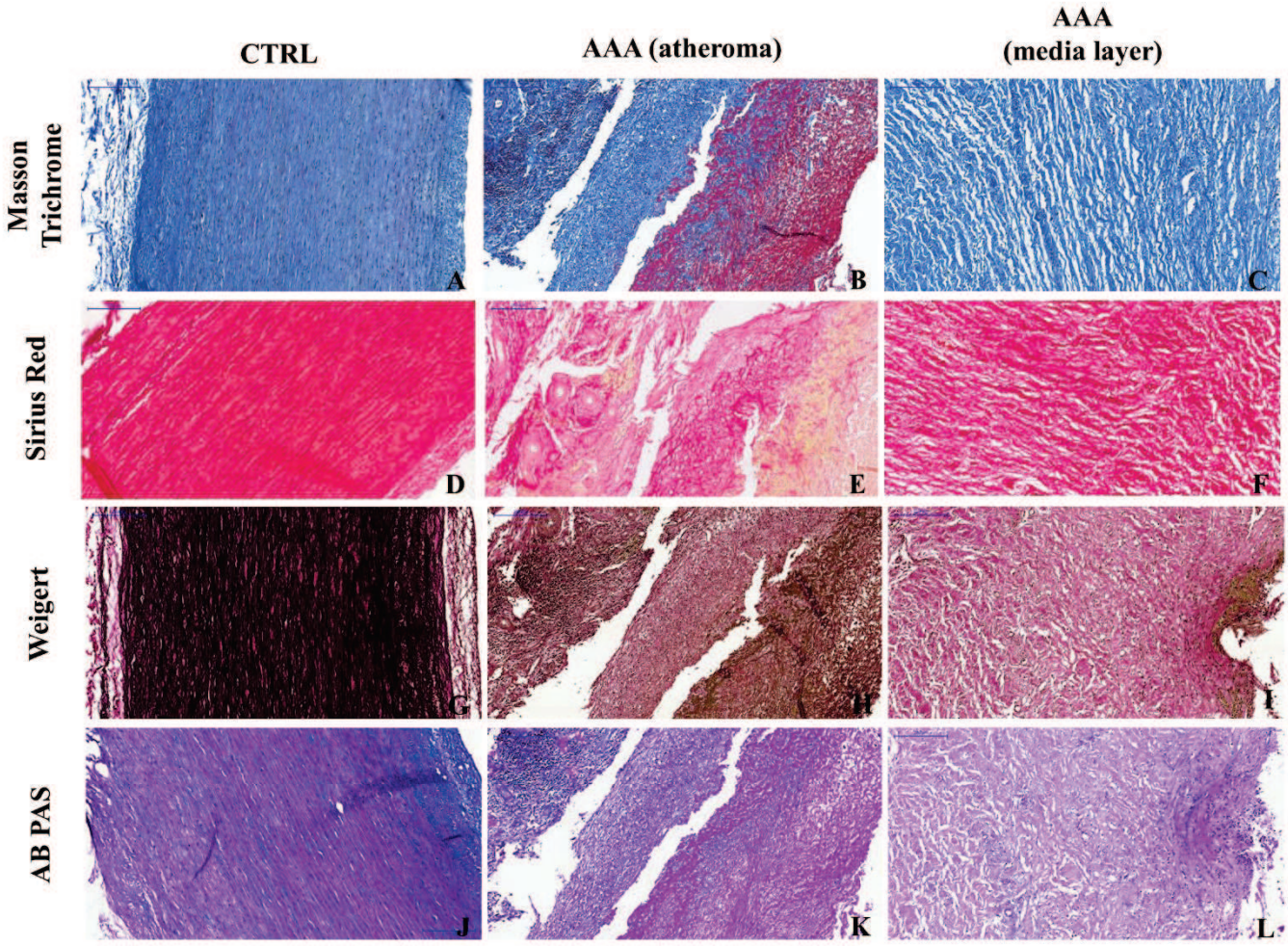

Figure 23. Representative histological stainings performed on control and AAA sections. Masson thricrome and sirius red show collagen contenent, Weigert is performed for elastic fibers and AB-pas for cystic medial necrosis. Scale bar 200µm

The thickening of aortic wall observed in presence of AAA decreases the amount of nutrients and oxigen that cells receive by diffusion, which is further accentuated by the presence of intra-luminal thrombus, leading to relative hypoxia. Neoangiogenesis occurs as a part of an adaptive response to survive in a relatively hypoxic environmental.

Indeed, medial neo-angiogenesis was found in the medial layer of aneurysm samples as shown by hematoxylin and eosin staining. (Figure 24) Interestingly, a strong positive C4d immunostaining, indicating that an inflammatory signal specific for the formed medial endothelial structure was present. (Figure 24) A unique feature of C4d is that it binds covalently to the endothelial and collagen basement membranes. Thus, the accumulation of C4d indicates a chronic inflammatory events sustained by ECs.

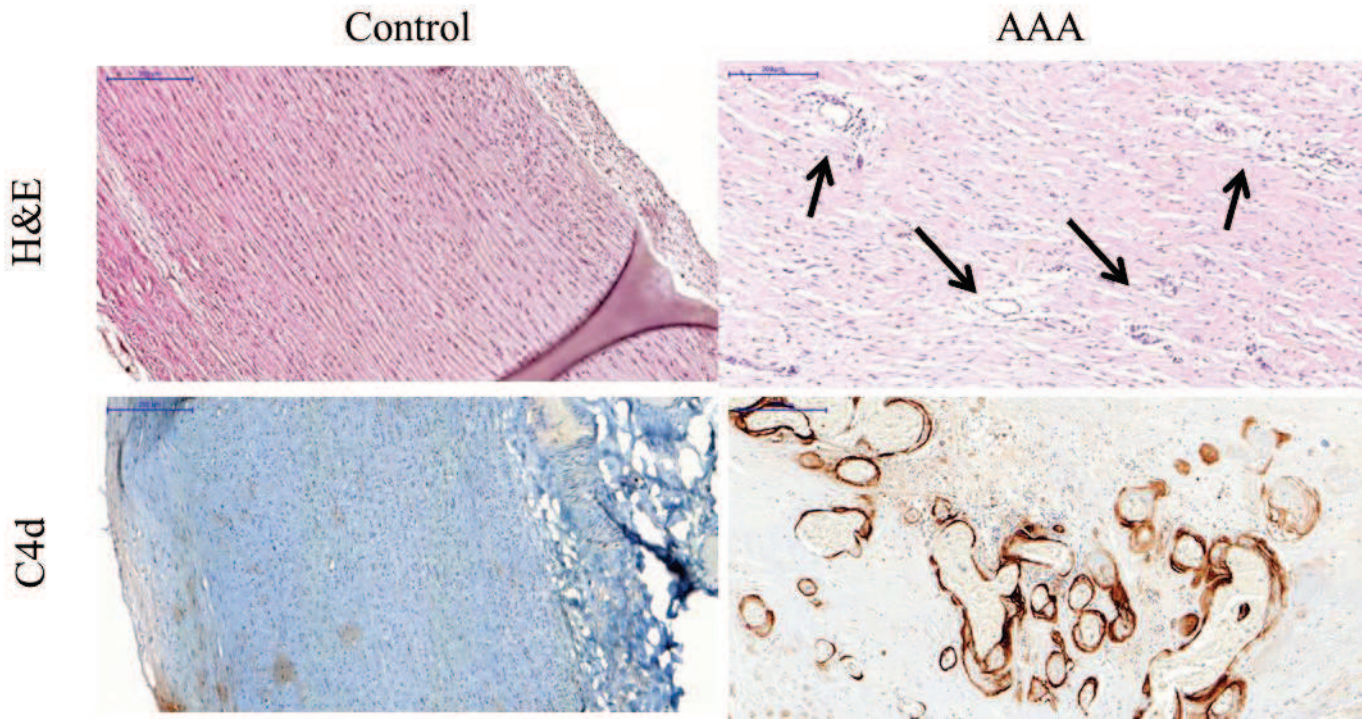

Figure 24. Representative hematoxylin-eosin performed on control and AAA: medial neo-angiogenesis is shown. (Arrows) Scale bar: 200 µm. Representative immunohistological staining for C4d. Scale bar: 200 µm

MCP-1, a monocyte chemotactic factor that signals via the CCR2 receptor, is critical for aneurysm formation because of its ability to recruit leukocytes that, in turn, produce ECM-degrading MMPs, thereby inducing aortic wall remodeling and dilatation.

Among vascular cytokines, IL-6 is well known to be secreted at high levels in human aortic aneurysm disease. It has been identified as an independent biomarker of severe coronary artery disease and it is associated with an increased risk for aneurysm rupture. In fact, aneurysms showed a significant upregulation of both, MCP-1 and IL-6 as shown by immunofluorescence stainings. (Figure 25) MCP-1 (red) was identified in both the endothelium and the medial layer. IL-6 (green) was predominantly detected in the adventitia, in proximity of *vasa vasorum*, and it was barely detectable in the media and intima. Abdominal aorta controls were negative for all these markers.

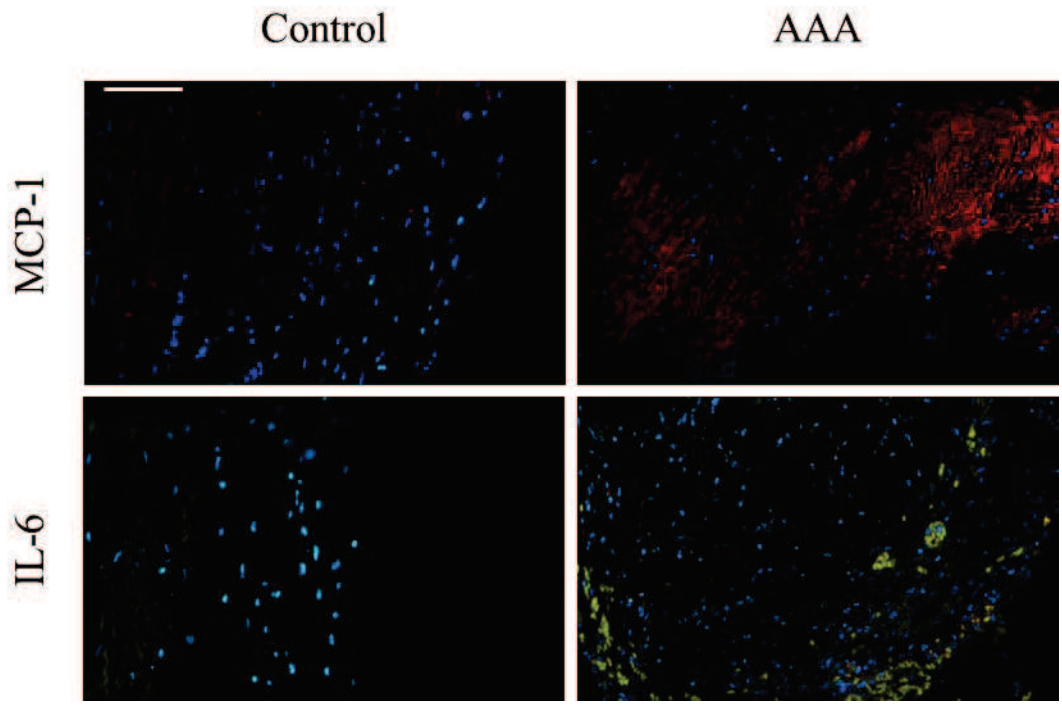

Figure 25. Representative immunofluorescence analysis for MCP-1 and IL-6 on control and AAA tissues. Scale bar: 200 µm

4.3 MMP-9 expression within healthy and AAA wall

MMP-9 involvement in pathological aortic remodeling contextually to AAA, was demonstrated by zymography assay. Zimography assay showed a significant increase in MMP-9 expression in aneurysmatic tissues compared to control. (Figure 26A) Aortic wall affected by aneurysm contains several cell types as possible source of MMP-9. As expected, high expression of MMP-9 in serial sections of AAA specimens, but not in normal tissue, were observed. (Figure 26B) In these sections, MMP-9 was detected in the inflammatory infiltrate. The high concentration of inflammatory cells observed in these specimens was probably due to the transmural migration of circulating cells in response to chemotactic factors; moreover, vascular resident cells greatly contribute to the secretion of MMPs, in particular MMP-9. In good agreement with this, in medial neo-angiogenesis, where ECs are present, MMP-9 expression was observed. (Figure 26B) As previously shown, the aneurysmatic vessel wall microstructure resulted significanlty altered, and neo-aniogenesis was evident in the medial layer.

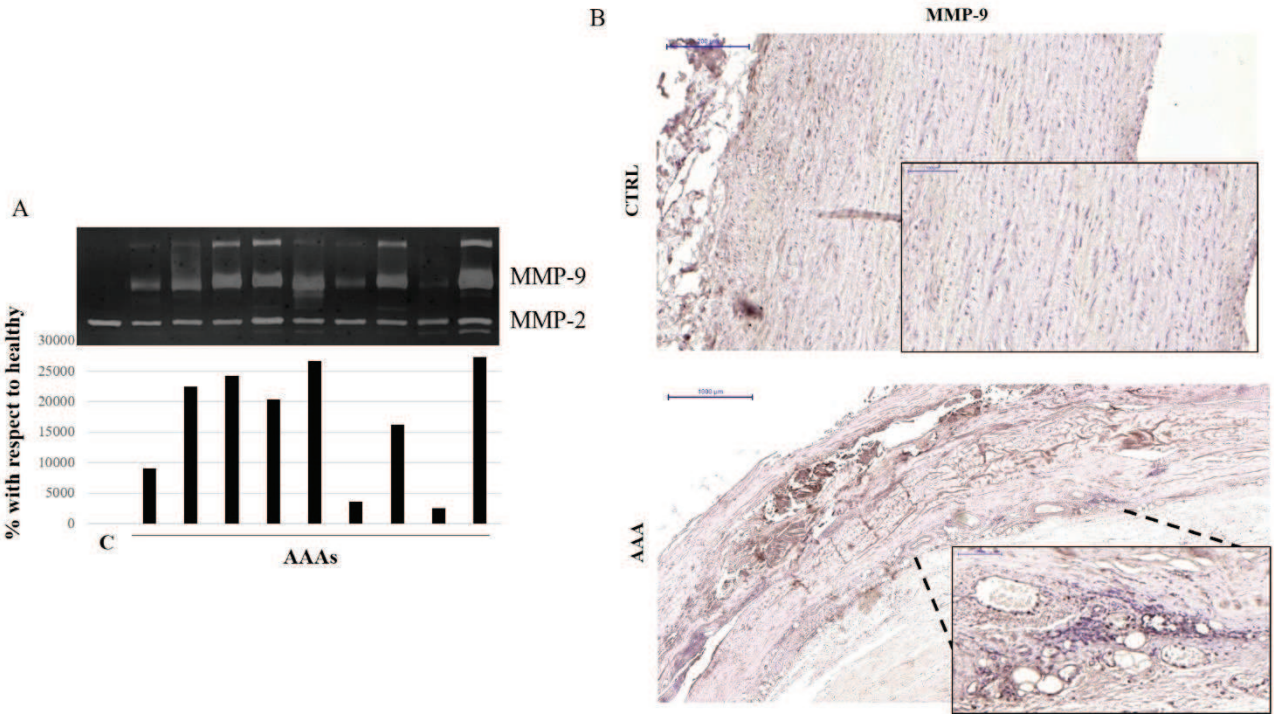

Figure 26. A) Representative gelatin zymography assay and relative densitometry on fresh tissues (control and AAAs from different patients) n value=3. B) Representative immunohistochemistry for MMP-9 on control tissue. Scale bar: 200 µm and 100 µm and representative immunohistochemistry on AAAs. Scale bar: 1000 µm and 100 µm.

4.4 *In vitro* model of ECs silenced for MMP-9

Since ECs play a key role in wall dysfunction, and previous data on human samples confirmed the role of MMP-9 in aneurysm development, we sought to determine whether lack of endothelial MMP-9 could affect EC-mediated matrix remodeling. For this purpose, we silenced MMP-9 gene expression in EA.hy926 cells using lentiviral-transduced shRNA. First, EA.hy926 cells were co-transduced with a combination of various LVs: shRNA1+shRNA1; shRNA1+shRNA5; shRNA5+shRNA5, and shRNA5+shRNA1. Subsequently, MMP-9 protein expression was measured in transduced cells treated in presence of TNF-α (50ng/mL). To compare the expression of MMP-9 in cell lysates and in supernatants, immunoblotting and zymography assay (Figure 27A and Figure 27B respectively) were performed. As expected, a significant downregulation of both proMMP-9 and MMP-9 in lysates of shRNA5- and/or shRNA1-transduced EA.hy926 cells compared to control cells (Ctrl) was observed (Figure 27A). Likewise, secreted MMP-9 was significantly down-regulated following MMP-9 gene silencing, albeit to different extents (Figure 27B). Moreover, after TNF-α treatment wild-type EA.hy926 showed a significant upregulation of MMP-9, which was strongly inhibited in shRNA5+shRNA1-transduced cells (Figure 27C).

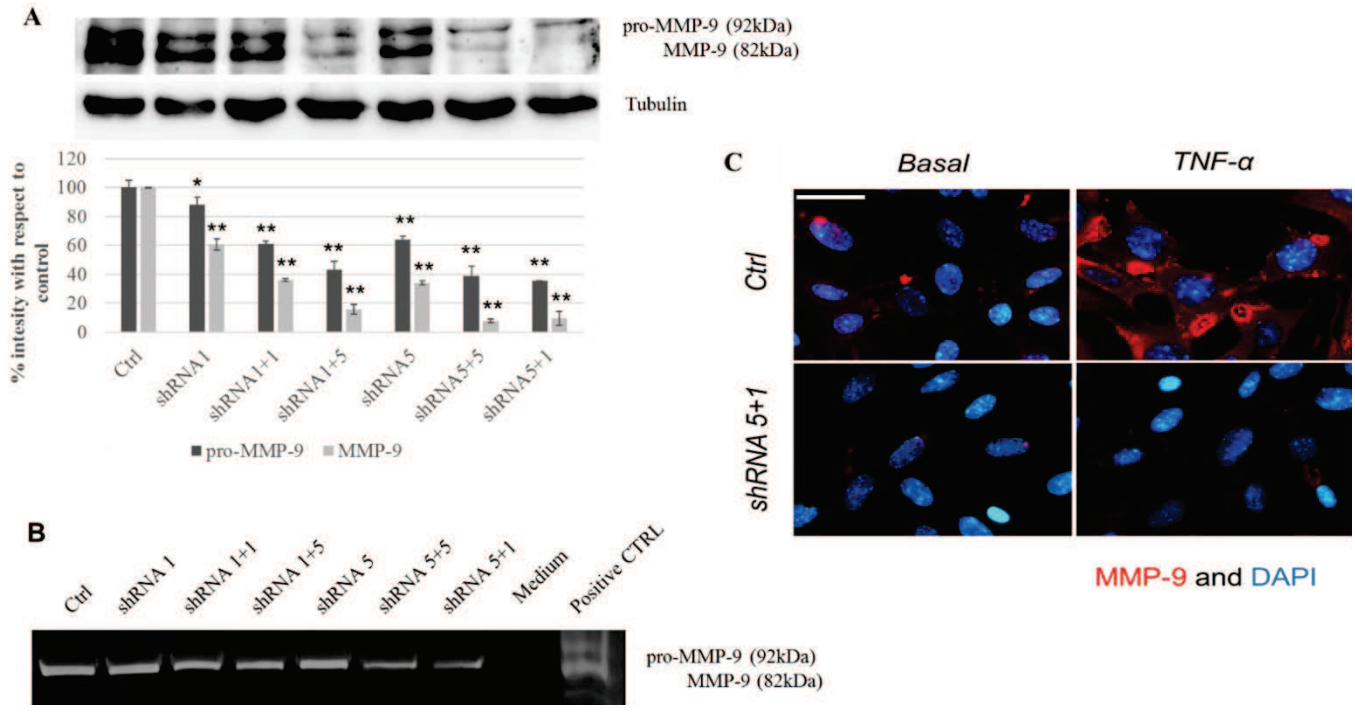

Figure 27. MMP-9 silenced EA.hy926 cells. A) Western blot and relative densitometry of control and shRNA lentiviral-transduced EA.hy926 cells using antibodies against MMP-9 and tubulin. Relative densitometry value s obtained from three different experiments are expressed as mean±SD. n value=3. * indicates p≤0,05; ** ≤0,001.
B) Zymography assay performed on control and transduced EA.hy926 endothelial cells stimulated with human recombinant TNF-α (50ng/mL). Peripheral blood was used as standard. n value =3. C) Representative immunofluorescence staining for MMP-9 on wild type and transduced cell. Basal control (without TNF-α) did not express MMP-9. n value=3. Scale bar: 40 µm

4.5 Tube formation assay

We assessed the ability of ECs void of MMP-9 to promote tube formation *in vitro*. When wild-type EA.hy926 cells were placed on growth factor-reduced Matrigel upon VEGF stimulation, elongated, cross-linked, and robust cord-like structures were observed. Remarkably, VEGF-induced tube formation was strongly impaired in EA.hy926 lacking MMP-9. (Figure 28) Thus, data indicate that MMP-9 is required for EA.hy926 cell migration and angiogenesis.

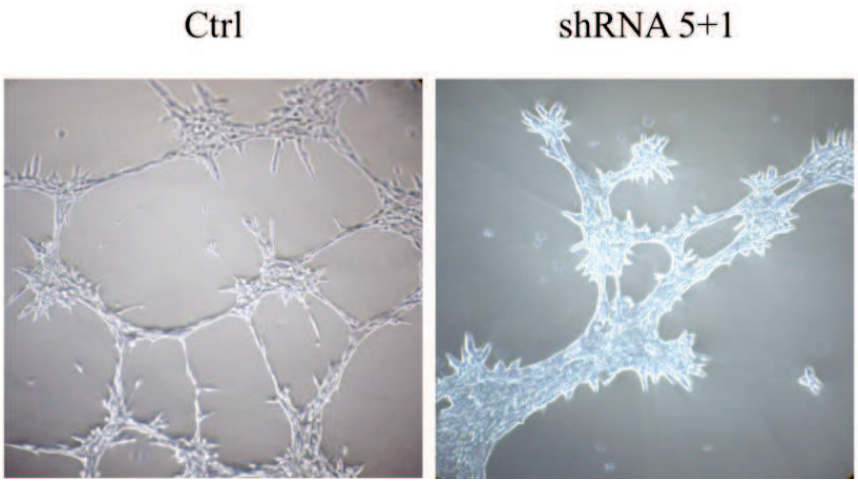

Figure 28. Tube formation assay in Matrigel matrix.

4.6 NF-kB activation

Since TNF-α induces the inflammatory response through NF-kB activation in ECs, we sought to determine whether MMP-9 silencing could affect TNF-α-mediated induction of NF-kB. As expected, an early and transient upregulation of phospho-p65 protein in wild-type EA.hy926 cells treated with TNF-α was observed, whereas p65 remained unaltered. In contrast, in EA.hy926 shRNA5+shRNA1-transduced cells, TNF-α-mediated phosphorylation of p65 was completely inhibited. (Figure 29)

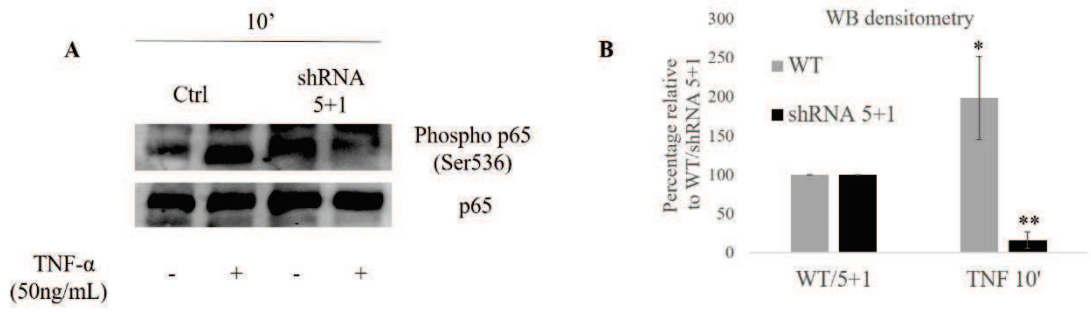

Figure 29. NF-kB activation. Western blot and relative densitometry for phospho p65 and p65 expression. * indicates p≤0,05; ** ≤0,001.

Western blot was further confirmed by immunofluorescence assay where a marked nuclear translocation was shown after 10 minutes in wild type cells treated with TNF-α. (Figure 30) After 30 minutes, a decrease of nuclear positive staining was observed, while no nuclear p-65 signal was present after 60 minutes. On the other hand, in MMP-9 silenced cells no p-65 nuclear translocation was observed. (Figure 30)

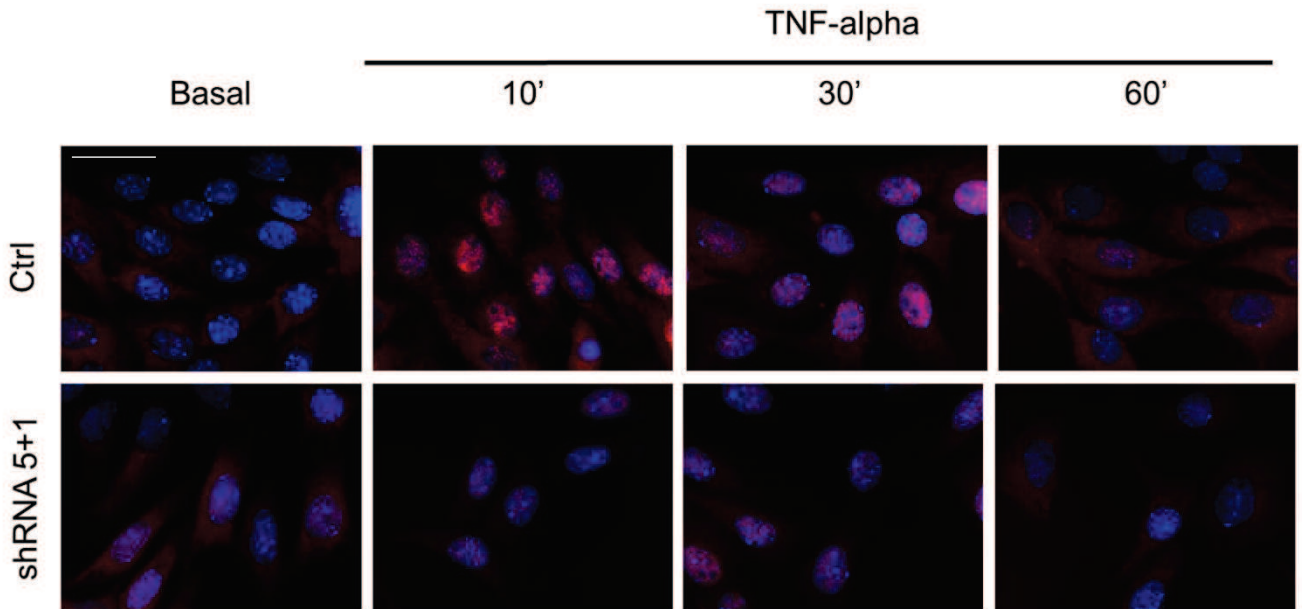

Figure 30. Representative immunofluorescence staining for p-65. n value=3. Scale bar: 40 μm

4.7 MMP-9 regulates cytokines production by ECs and inflammatory cells adhesion

In order to determine whether endothelial-produced MMP-9 may promote a pro-inflammatory autocrine loop through regulation of cytokine expression, cytokine antibody arrays on conditioned media from WT- or shRNA5+shRNA1-transduced cells were performed. Briefly, following 24h incubation in free-FBS medium in the presence or absence of TNF-α (50ng/mL) the conditioned media were collected. In the conditioned media derived from MMP-9-silenced EA.hy926 cells, reduced expression of several cytokines and growth factors was observed: GM-CSF (16%), IL-3 (8%), IL-5 (32%), IL-6 (34%), IL-7 (38%), IL-8 (9%), MCP-2 (41%) and MCP-3 (30%), where each percentage in bracket represents cytokine expression relative to non-transduced EA.hy926 control cells. (Figure 31A)

To validate cytokine array data, immunofluorescence assay on cells similarly treated was performed. In wt-EA.hy926-treated cells, an upregulation of MCP-1 expression was observed, whereas in transduced cells was significantly reduced. (Figure 31B) Likewise, TNF-α-mediated

activation of E-selectin (CD62E) was inhibited in MMP-9-silenced EA.hy926 cells, but not wild type cells.

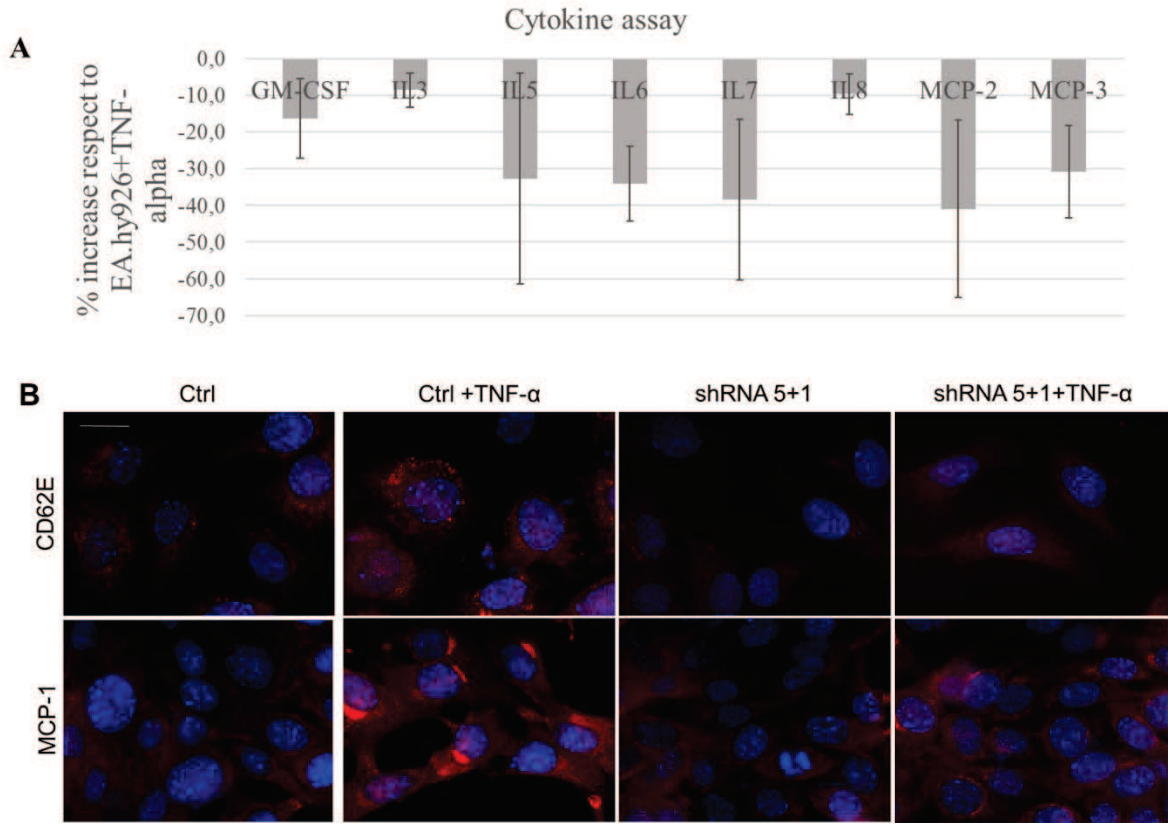

Figure 31. MMP-9 affects the inflammatory response in ECs. A) Cytokines assay performed on conditioned medium (CM) obtained from wild type and transduced (shRNA 5+1) endothelial cells treated with TNF-α in starving conditions. n value=3 B) Representative immunofluorescence for E-selectin (CD62E) and MCP-1 on wild type and transduced EA.hy926. . n value=3

Since CD62E plays an important role in leukocytes recruiting, the effects of lack of MMP-9 on U937 cell adhesion was determined. Indeed, MMP-9 silenced cells showed a 3-fold decrease in U937 adhesion in response to TNF-α treatment compared to wild-type cells similarly stimulated. Thus, lack of endothelial MMP-9 affects EC-mediated secretion of pro-inflammatory cytokines.

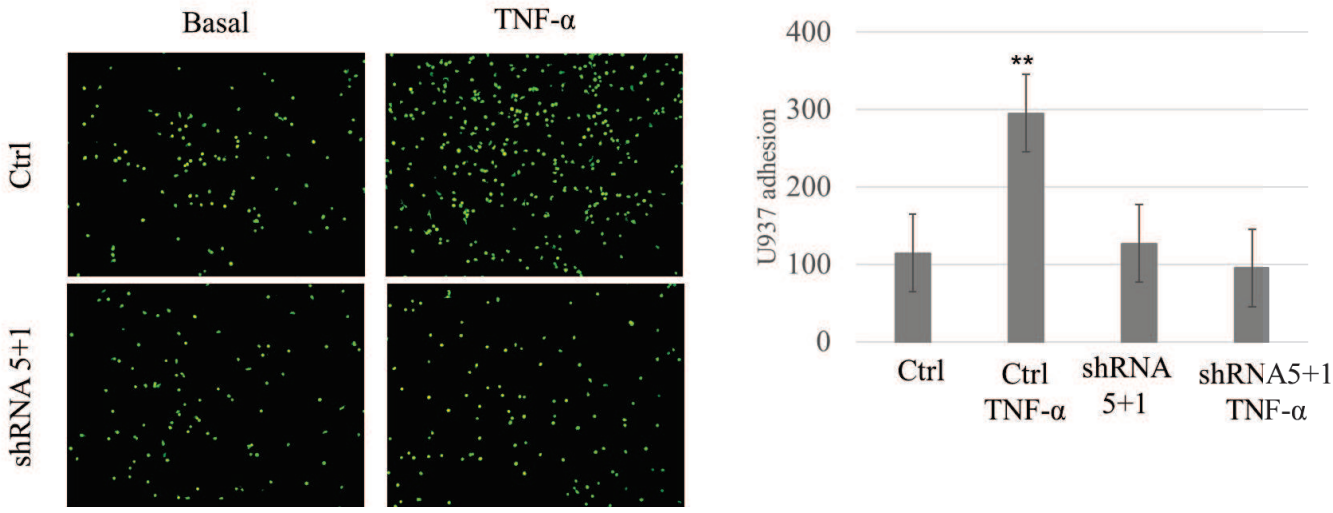

Figure 32. U937 adhesion on ECs. * indicates ** ≤0,001. n value = 3

4.8 Effect of conditioned media (CM) on vascular smooth muscle cells (vSMC) and macrophages

In order to characterize the role of MMP-9 in the interaction between ECs, vSMCs and macrophages, the effect of conditioned medium (CM) obtained from wt- or shRNA 5+1-transduced EA.hy926 cells on vSMC pro-angiogenic functions and on macrophages was assessed.

First, MMP-9 expression in vSMCs cultured in either wt- or transduced endothelial cell-derived CM was measured. As negative control, lysates from vSMCs grown in non-conditioned medium (basal) were similarly isolated. (Figure 33) As expected, MMP-9 in vSMCs cultured in basal medium could not be detected. (Figure 33)

Interestingly, vSMCs grown in EA.hy926 cell-derived CM showed enhanced MMP-9 expression as compared to cells grown with basal medium, suggesting that EA.hy926 cells secrete pro-angiogenic factors able to induce MMP-9 expression. (Figure 33) Intriguingly, also lysates from vSMCs cultured with shRNA5+1-transduced EA.hy926 cells showed higher MMP-9 expression than basal, implying that this effect is independent from MMP-9 expression in EA.hy926 cells. When MMP-9 expression was measured in vSMCs grown in CM obtained from wt-EA.hy926 cells treated for 24h with TNF-α, further upregulation of MMP-9 expression was observed. (Figure 33) In contrast, when CM obtained from MMP-9-silenced EA.hy926 cells is used, TNF-α-mediated activation of MMP-9 was almost inhibited (Figure 32), suggesting that MMP-9 expression in EA.hy926 cells mediates, in part, vSMC-mediated angiogenesis. To further address this hypothesis, the extent of proteolytic cleavage of elastin in the different conditioned and basal media was compared. Remarkably, TNF-α treatment induced proteolytic cleavage of elastin in medium from vSMCs

cultured with wild-type EA.hy926 cell-derived CM, but not shRNA 5+1 transduced cell-derived CM, suggesting that endothelial MMP-9 silencing ultimately inhibits elastin fragmentation also in pro-inflammatory conditions. (Figure 33)

IL-6, it is highly modulated by the presence of TNF-α. However, wild-type endothelial CM did not show any significant effect with respect to basal medium, indicating that no pro-inflammatory stimuli were present. In fact, when in presence of TNF-α, IL-6 resulted significantly up-regulated. (Figure 33B) Moreover, data suggest that CM of wild type EC, enriched with TNF-α, contains more pro-inflammatory mediators with respect to CM of MMP-9 silenced cells enriched with TNF-α. In fact, in this case, IL-6 resulted significantly decreased with respect to CM obtained from wild-type ECs. Moreover, together with MMP-9 up-regulation, vSMCs cultured with wild type CM enriched with TNF-α showed a proteolytic elastin capability. In fact, only in the lane corresponding to vSMCs cultured with CM containing TNF-α obtained from wild type ECs showed a smear, comparable with the lane corresponding to elastin digested with elastase. (Arrow in Figure 33C)

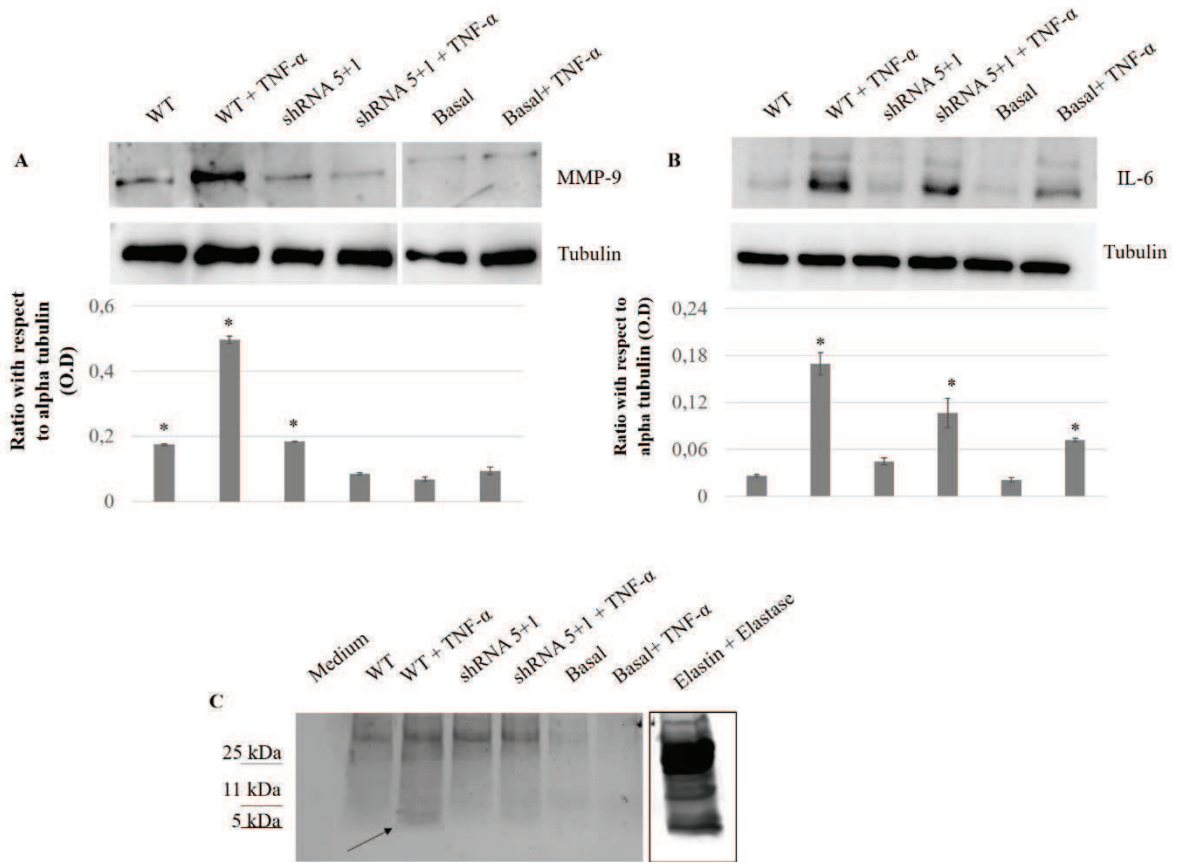

Figure 33. A) Western blot on MMP-9 and relative densitometry performed on vSMCs cultured with CM obtained from wild type and transduced EA.hy926 endothelial cells treated TNF-α (50ng/mL) Significance has been evaluated with respect to basal culture conditions with p≤0,001. B) Western blot on IL-6 and relative densitometry performed on vSMCs cultured with CM obtained from wild type and transduced EA.hy926 endothelial cells treated TNF-α (50ng/mL) Significance has been evaluated with respect to basal culture conditions with p≤0,001. C) Silver staining on cell culture media. Elastin digested with elastase has been used as standard.

Finally, macrophages have been cultured for 24h with Conditioned Medium (CM) obtained from wild-type and transduced (shRNA 5+1) endothelial cells. Macrophage phenotype has been characterized by specific surface markers such as CD14, CD11b and CD105. (Figure 34B) Macrophages maintained with CM obtained from ECs for 24 hours, showed no differences related to MMP-9 and IL-6 expression with respect to basal, also in presence of CM obtained from transduced cells. (Figure 34C and 34D) These data showed that endothelial MMP-9-silenced has a specific effect on vSMCs, without affecting macrophages behavior.

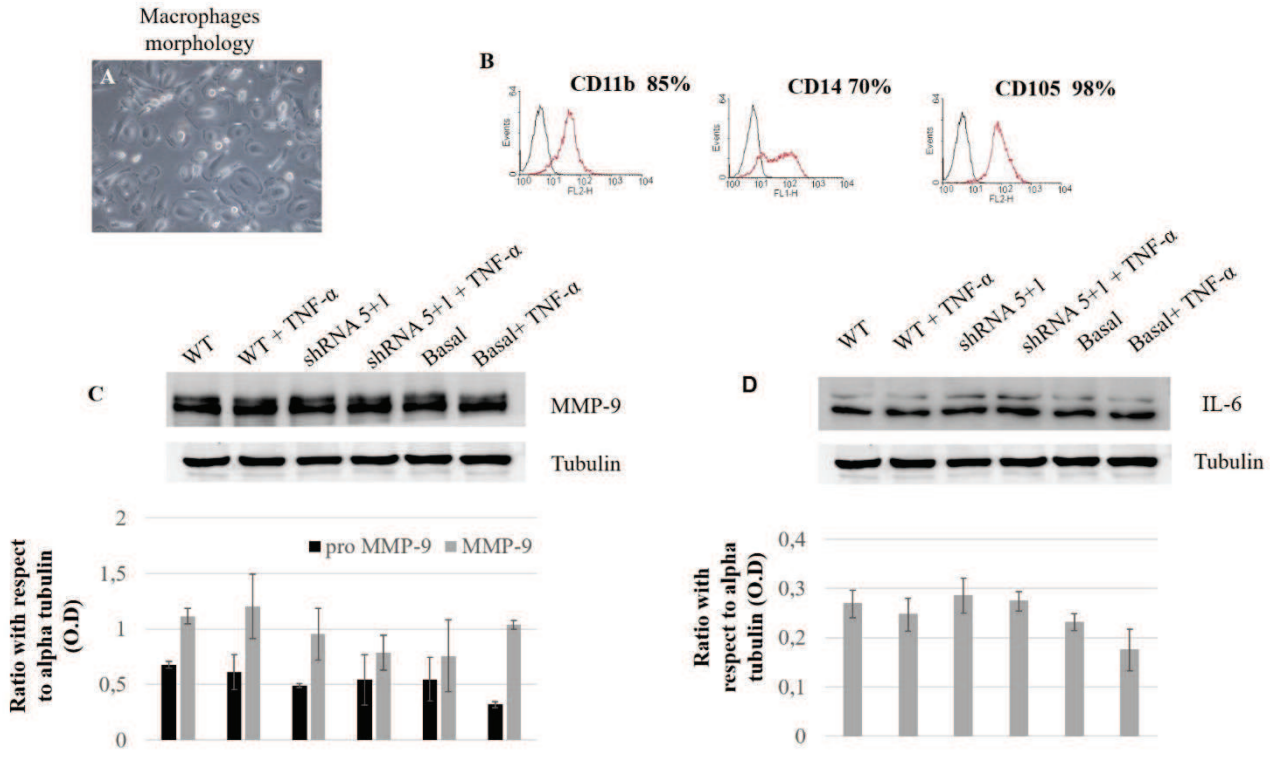

Figure 34. A) Macrophages morphology after 7 days of differentiation media. B) FACS analysis for macrophages markers C) Western blot on MMP-9 and relative densitometry performed on macrophages cultured with CM obtained from wild type and trasducted EA.hy926 endothelial cells treated TNF-α (50ng/mL) Significance has been evaluated with respect to basal culture conditions with p≤0,001. B) Western blot on IL-6 and relative densitometry performed on macrophages cultured with CM obtained from wild type and trasducted EA.hy926 endothelial cells treated TNF-α (50ng/mL) Significance has been evaluated with respect to basal culture conditions with p≤0,05.

5. Discussion

Vascular diseases display a heterogeneous localization along the aorta. Aortic aneurysms can develop in both the thoracic (TAA) and abdominal aorta (AAA). However, TAA and AAA have pathogenesis. AAA has a higher incidence with respect to TAA. Moreover, the majority of these aneurysms have an atherosclerotic nature. (Ladich et al., 2016) In fact, abdominal aortic media has peculiar morphological characteristics, consisting in a decreased elastin content corresponding to a reduced number of lamellar units (28-32), in comparison to the thoracic segment (55-60). This aspect makes abdominal aorta more susceptible to aneurysm event. (Ruddy et al, 2008)

AAA is partially characterized by dramatic modifications of the endothelial, medial and adventitia layers (Daugherty et al., 2000). These modifications are: presence of intraluminal thrombus, calcification, inflammatory cell infiltrate, loss of medial vSMC phenotype, elastin degradation and collagen replacement resulting in a thin fibrotic wall. (F. A. M. V. I. Hellenthal et al., 2009) In particular, in the medial layer, AAA is histologically typified by vSMC depletion and degradation of ECM (loss of alpha-smooth muscle actin-positive cells in aneurysm tissues respect to healthy aortas). Elastin content is limited, structure results fragmented and altered, while there is high collagen content.

In human aorta, ECM turnover is a physiologic process, necessary for the maintenance of tissue homeostasis. This physiological remodeling is mediated by proteolytic enzymes, among which MMPs are the most characterized and studied. However, MMPs are also responsible for ECM degradation during tissue remodeling when in presence of pathological conditions. Several MMPs have been studied in relation to AAA, such as MMP-1, MMP-2, MMP-3, MMP-9, MMP-12 and their inhibitor called TIMPs. MMP-9 robustly increases during several cardiovascular diseases, including hypertensions, atherosclerosis and myocardial infarction. (Yabluchanskiy et al., 2013) In particular, in AAA, MMP-9 stimulates the immune response to initiate pathogenesis and exacerbate the disease progression through proteolytic degradation of proteins in the basal lamina of blood vessels as well as the release of the biologically active form of VEGF. (Bergers et al., 2000) Furthermore, MMP-9 serum levels significantly correlate with aneurysmal tissues. (Stather et al., 2014)

AAA is histologically characterized by an accumulation of inflammatory cells, (Wang et al., 2014) and the inflammatory process contributes to a loss of contractile SMC phenotype. (Beamish et al., 2010) In particular, the presence of inflammatory cells is correlated to high level of IL-6 and MCP-1. Moehle et al. showed that MCP-1 is required for AAA formation in terms of macrophages recruitment and vSMC phenotype modulation in mice. (Moehle et al., 2011) Wang et al. demonstrated recombinant MCP-1 induced IL-6 expression in human macrophages, and IL-6 exposure time-

dependently induces vSMC apoptosis via Stat1. In this context they found a MCP-1/IL-6 regulatory loop between vSMC and macrophages.(Wang et al., 2015)

Medial neo-angiogenesis, which leads to hyper-permeable vessels together with ECM degradation, indicates that endothelial cells play a key role in wall dysfunctions. (Jones, 2011) However, the specific role of ECs still remains poorly understood. The observation that medial neo-angiogenesis leads to increased vascular permeability and ECM degradation indicates that ECs play a key role in wall dysfunction. Furthermore, during neo-angiogenesis within the medial layer, human AAA expresses high levels of C4d, a degradation product of the classic complement pathway, which covalently binds to the endothelial and collagen basement membranes. (Josephson, 2014) Thus, the accumulation of C4d is a hallmark of EC chronic inflammation.

In order to better understanding the role of ECs and the role of endothelial MMP-9 in AAA progression, EA.hy926 cells silenced for MMP-9 have been used. MMP-9 silencing was performed through short hairpin-RNA (shRNA) delivered by lentiviral vector (LVs) which represent a valuable tool for gene therapy in that they are able to transduce non-dividing cells, integrate into the genome of target cell with high levels and long-term transgene expression and accommodate relatively large genes. RNA interference (iRNA) is a potent and specific mechanism for regulating gene expression whose inhibitory effect results from selective degradation of target mRNA. (McManus and Sharp, 2002) Data clearly showed that tube formation activity of MMP-9-silenced EA.hy926 cells is impaired, implying that MMP-9 is required for matrix remodeling.

Previous data showed that the recruitment of inflammatory cells is sustained by a chemotactic gradient due to cytokines released through EC activity, and by the increased expression of adhesion molecules able to interact with circulating inflammatory cells. These cytokines include TNF-α, ILs, lymphokines, monokines, interferons, colony stimulating factors, and transforming growth factors, although these cytokines are produced by cell types other than ECs, such as macrophages, T cells and monocytes, as well as platelets and VSMCs. (Sprague and Khalil, 2009) Among these inflammation mediators, TNF-α plays a pivotal role in the initiation and progression of vascular disorder by modulating the expression of molecules involved in vascular tone, inflammation and remodeling, thus, inducing endothelial dysfunction. (Zhang et al., 2009) (Palmieri et al., 2014) In particular, TNF-α-mediated activation of NF-kB has been shown to be one of the major pathway contributing to inflammation-mediated tissue damage. Thus, the goal of this work has been investigating whether lack of endothelial MMP-9 could impair TNF-α/NF-kB signalling pathway. Data clearly showed that both TNF-α-mediated phosphorylation of p65 and p65 nuclear translocation, characteristics of NF-kB activation, were significantly decreased in EA.hy926 cells silenced for MMP-9. Thus, MMP-9

plays a role during TNF-α-mediated activation of NF-kB. Remarkably, MMP-9 silencing showed an autocrine effect on ECs since several cytokines resulted down-regulated, especially IL-6, IL-7, MCP-1. As it is well-established that TNF-α-mediated upregulation of these cytokines requires unaltered NF-kB signaling, these findings indicate that also MMP-9 regulate cytokine production in ECs during matrix remodeling. Data show also that lack of endothelial MMP-9 affects TNF-α-mediated upregulation of CD62E, a selectin that mediates leukocyte cell adhesion to activated platelets and other leukocytes in response to TNF-α, IL-1β or LPS**, t**hereby initiating multicellular adhesive and signaling events during physiological or pathological inflammation processes. (McEver, 2015) Thus, data indicating the requirement of MMP-9 for TNF-α-mediated upregulation of CD62E consistent with the observation that MMP-9 released from ECs represents a fundamental switch able to control the inflammatory response. In this regard, MMP-9 has been shown to release active TNF-α from the cell surface via proteolysis. (Gearing et al., 1994)

Results also support the knowledge that TNF-α mediates the inflammatory response in ECs. TNF-α is an activator of NF-kB which plays a central role in the development of the inflammatory response through further regulation of genes encoding not only pro-inflammatory cytokines, but also adhesion molecules, such as CD62E, VCAM-1 and ICAM-1, chemokines, growth factors, and inducible enzymes such as cyclooxygenase-2 (COX2) and inducible nitric oxide synthase (iNOS). NF-κB is also required for cytokine upregulation of MMP-1, -3 and -9. (Bond et al., 2001)

Since the vascular inflammatory response involves complex interaction among white cells (i.e. neutrophil, lymphocytes, monocytes and macrophages) ECs and vSMC, the influence of MMP-9 secreted by ECs on vSMCs competence in vessel wall matrix remodeling and weakening was assessed. In literature, two different co-culture methods outlined from several studies on atherosclerosis. Indirect co-cultures are useful to investigate cell responses between different cell types that involve secretory pathways and cytokine production. They are widely used to study different diseases through *in vitro* models. The main variations of the indirect co-culture models are microcarrier, scaffold, bilayer, conditioned media (CM) and trans-well methods. In this work, indirect co-cultures were performed. In particular, previous reports have shown that co-culturing of human aortic ECs and vSMC along with monocytes led to increased enrichment in cell culture medium of matrix remodeling effectors such as fibronectin, collagen, IL-1 and IL-6. (Ghosh et al., 2015)

Data show that CM obtained from ECs cultured in the presence of TNF-α upregulate MMP-9 expression level in vSMCs, suggesting that this CM contains a sufficient amount of inflammatory molecules able to stimulate vSMCs functions. Interestingly, treatment of vSMCs with CM derived from MMP-9-silenced EA.hy926, exposed to TNF-α, led to a dramatic inhibition of MMP-9

expression levels. This finding might be explained, in part, by the assumption that CM derived from MMP-9-silenced EA.hy926 cells contains lower levels of cytokines, growth factors, and adhesion molecules required for EC-mediated activation of vSMC functions during neo-angiogenesis. In support to this conclusion, CM from MMP-9-silenced EA.hy926 cell, but not wild type cells, did not display proteolytic activity in terms of elastin degradation *in vitro*.

In blood vessels, elastin is synthetized and secreted by vSMCs. (Patel et al., 2006) In the event of injury, the production of tropoelastin can be quickly restored and it is influenced by several exogenous factors such as TNF-α, IL-1β, insulin-like growth factor-1 and strongly by transforming growth factor. (Pierce et al., 2006) MMPs are particularly important in elastin breakdown, and MMP-2, -3, -9 and -12 can directly degrade elastin. (Ra and Parks, 2007) Moreover, elastin fragments promote monocyte/macrophage recruitment *in vivo*.(Adair-Kirk and Senior, 2008) Thus, consistently these findings, data suggest that ECs have a humoral cross-talk with vSMCs, that can locally sustain the pathogenesis of AAA, and open new scenarios concerning the role of EC-produced MMP-9 in AAA development.

Although, these data demonstrate that medial layer ECs play a key role in the pathogenesis and maintenance of AAA *in situ* through regulation of vSMC functions, which ultimately promotes ECM damage. Importantly, the fact that MMP-9-silenced ECs are no longer able to migrate and differentiate in a three dimensional matrix strongly indicates that MMP-9 regulates proangiogenic EC functions as well.

In conclusion, the experimental approach used in this study opens new perspective for the treatment of AAA. In this regard, the advancement of more than 20 therapeutic siRNAs into the clinic indicates that RNAi-based medicine holds promise as future therapeutic option for AAA. However, several obstacles in clinical development of RNAi-based therapeutics, foremost the delivery methods to target cells, are yet to be overcome. (Burnett et al., 2011)

6. References

Adair-Kirk, T.L., Senior, R.M., 2008. Fragments of extracellular matrix as mediators of inflammation. International Journal of Biochemistry and Cell Biology.

Alexander, J.P., Acott, T.S., 2001. Involvement of protein kinase C in TNFalpha regulation of trabecular matrix metalloproteinases and TIMPs. Investigative ophthalmology & visual science 42, 2831–2838.

Arias-Salgado, E.G., Lizano, S., Sarkar, S., Brugge, J.S., Ginsberg, M.H., Shattil, S.J., 2003. Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. Proceedings of the National Academy of Sciences of the United States of America 100, 13298–13302.

Arribas, S.M., Hinek, A., González, M.C., 2006. Elastic fibres and vascular structure in hypertension. Pharmacology and Therapeutics.

Bagowski, C.P., Ferrell, J.E., 2001. Bistability in the JNK cascade. Current Biology 11, 1176–1182.

Baum, R.A., Stavropoulos, S.W., Fairman, R.M., Carpenter, J.P., 2003. Endoleaks after endovascular repair of abdominal aortic aneurysms. Journal of vascular and interventional radiology : JVIR 14, 1111–1117.

Baxter, B.T., Pearce, W.H., Waltke, E.A., Littooy, F.N., Hallett, J.W., Kent, K.C., Upchurch, G.R., Chaikof, E.L., Mills, J.L., Fleckten, B., Longo, G.M., Lee, J.K., Thompson, R.W., 2002. Prolonged administration of doxycycline in patients with small asymptomatic abdominal aortic aneurysms: Report of a prospective (Phase II) multicenter study. Journal of Vascular Surgery 36, 1–12.

Bazzoni, G., Dejana, E., 2004. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. Physiol Rev. 84, 869–901.

Beamish, J.A., He, P., Kottke-Marchant, K., Marchant, R.E., 2010. Molecular Regulation of Contractile Smooth Muscle Cell Phenotype: Implications for Vascular Tissue Engineering. Tissue Engineering Part B: Reviews 16, 467–491.

Benbow, U., Brinckerhoff, C.E., 1997. The AP-1 site and MMP gene regulation: What is all the fuss about? Matrix Biology 15, 519–526.

Bergers, G., Brekken, R., McMahon, G., Vu, T.H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z., Hanahan, D., 2000. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nature cell biology 2, 737–44.

Boccafoschi, F., Mosca, C., Bosetti, M., Cannas, M., 2011. The role of mechanical stretching in the

activation and localization of adhesion proteins and related intracellular molecules. Journal of Cellular Biochemistry 112, 1403–1409.

Bonauer, A., Boon, R. a, Dimmeler, S., 2010. Vascular microRNAs. Current drug targets 11, 943–9.

Bond, M., Chase, A.J., Baker, A.H., Newby, A.C., 2001a. Inhibition of transcription factor NF-kappaB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. Cardiovascular research 50, 556–565.

Bond, M., Chase, A.J., Baker, A.H., Newby, A.C., 2001b. Inhibition of transcription factor NF-kappaB reduces matrix metalloproteinase-1, -3 and -9 production by vascular smooth muscle cells. Cardiovascular research 50, 556–565.

Boon, R.A., Seeger, T., Heydt, S., Fischer, A., Hergenreider, E., Horrevoets, A.J.G., Vinciguerra, M., Rosenthal, N., Sciacca, S., Pilato, M., Van Heijningen, P., Essers, J., Brandes, R.P., Zeiher, A.M., Dimmeler, S., 2011. MicroRNA-29 in aortic dilation: Implications for aneurysm formation. Circulation Research 109, 1115–1119.

Bourguignon, L.Y.W., Gunja-Smith, Z., Iida, N., Zhu, H.B., Young, L.J.T., Muller, W.J., Cardiff, R.D., 1998. CD44v3,8-10 is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. Journal of Cellular Physiology 176, 206–215.

Brew, K., Nagase, H., 2010. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochimica et biophysica acta 1803, 55–71.

Burnett, J.C., Rossi, J.J., Tiemann, K., 2011. Current progress of siRNA/shRNA therapeutics in clinical trials. Biotechnology journal 6, 1130–1146.

Caputo, M., Saif, J., Rajakaruna, C., Brooks, M., Angelini, G.D., Emanueli, C., 2015. MicroRNAs in vascular tissue engineering and post-ischemic neovascularization. Advanced Drug Delivery Reviews.

Cervantes, J., Martinez, R., Perez-Garcia, D., 1985. Acute thrombosis of abdominal aortic aneurysm. An uncommon entity. Journal of Cardiovascular Surgery 26, 598–601.

Clark, J.M., Glagov, S., 2015. Transmural organization of the arterial media. The lamellar unit revisited. Arteriosclerosis (Dallas, Tex.) 5, 19–34.

Cohen, M., Meisser, A., Haenggeli, L., Bischof, P., 2006. Involvement of MAPK pathway in TNF-

alpha-induced MMP-9 expression in human trophoblastic cells. Molecular human reproduction 12, 225–232.

Cordes, K.R., Sheehy, N.T., White, M.P., Berry, E.C., Morton, S.U., Muth, A.N., Lee, T.-H., Miano, J.M., Ivey, K.N., Srivastava, D., 2009. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature 460, 705–710.

Daley, W.P., Peters, S.B., Larsen, M., 2008. Extracellular matrix dynamics in development and regenerative medicine. Journal of cell science 121, 255–64.

Danyi, P., Elefteriades, J.A., Jovin, I.S., 2011. Medical therapy of thoracic aortic aneurysms: are we there yet? Circulation 124, 1469–1476.

Daugherty, A., Manning, M.W., Cassis, L.A., 2000. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. Journal of Clinical Investigation 105, 1605–1612.

Davies, P.F., 1995. Flow-Mediated Endothelial Mechanotransduction. NIH Public Access 75, 519–560.

Donnini, S., Morbidelli, L., Taraboletti, G., Ziche, M., 2004. ERK1-2 and p38 MAPK regulate MMP/TIMP balance and function in response to thrombospondin-1 fragments in the microvascular endothelium. Life sciences 74, 2975–2985.

Dubick, M.A., Hunter, G.C., Casey, S.M., Keen, C.L., 1987. Aortic ascorbic acid, trace elements, and superoxide dismutase activity in human aneurysmal and occlusive disease. Proc Soc Exp Biol Med 184, 138–143.

DUBOST, C., ALLARY, M., OECONOMOS, N., 1952. Resection of an aneurysm of the abdominal aorta: reestablishment of the continuity by a preserved human arterial graft, with result after five months. A.M.A. archives of surgery 64, 405–408.

Duca, L., Blaise, S., Romier, B., Laffargue, M., Gayral, S., El Btaouri, H., Kawecki, C., Guillot, A., Martiny, L., Debelle, L., Maurice, P., 2016. Matrix ageing and vascular impacts: Focus on elastin fragmentation. Cardiovascular Research.

Duca, L., Floquet, N., Alix, A.J.P., Haye, B., Debelle, L., 2004. Elastin as a matrikine. Critical Reviews in Oncology/Hematology.

Dziembowska, M., Milek, J., Janusz, a., Rejmak, E., Romanowska, E., Gorkiewicz, T., Tiron, a., Bramham, C.R., Kaczmarek, L., 2012. Activity-Dependent Local Translation of Matrix

Metalloproteinase-9. Journal of Neuroscience 32, 14538–14547.

Ejiri, J., Inoue, N., Tsukube, T., Munezane, T., Hino, Y., Kobayashi, S., Hirata, K., Kawashima, S., Imajoh-Ohmi, S., Hayashi, Y., Yokozaki, H., Okita, Y., Yokoyama, M., 2003. Oxidative stress in the pathogenesis of thoracic aortic aneurysm: protective role of statin and angiotensin II type 1 receptor blocker. Cardiovasc.Res. 59, 988–996.

Eyre, D.R., Paz, M. a, Gallop, P.M., 1984. Cross-linking in collagen and elastin. Annual review of biochemistry 53, 717–748.

Fahem, A., Robinet, A., Cauchard, J.H., Duca, L., Soula-Rothhut, M., Rothhut, B., Soria, C., Guenounou, M., Hornebeck, W., Bellon, G., 2008. Elastokine-mediated up-regulation of MT1-MMP is triggered by nitric oxide in endothelial cells. International Journal of Biochemistry and Cell Biology 40, 1581–1596.

Fish, J.E., Santoro, M.M., Morton, S.U., Yu, S., Yeh, R.F., Wythe, J.D., Ivey, K.N., Bruneau, B.G., Stainier, D.Y.R., Srivastava, D., 2008. miR-126 Regulates Angiogenic Signaling and Vascular Integrity. Developmental Cell 15, 272–284.

Follenzi, A., Naldini, L., 2002. HIV-based vectors. Preparation and use. Methods in molecular medicine 69, 259–274.

Fridman, R., Toth, M., Pe??a, D., Mobashery, S., 1995. Activation of Progelatinase B (MMP-9) by Gelatinase A (MMP-2). Cancer Research 55, 2548–2555.

Gasser, T.C., Ogden, R.W., Holzapfel, G. a., 2006a. Hyperelastic modelling of arterial layers with distributed collagen fibre orientations. Journal of the Royal Society, Interface / the Royal Society 3, 15–35.

Gasser, T.C., Ogden, R.W., Holzapfel, G.A., 2006b. Hyperelastic modelling of arterial layers with distributed collagen fibre orientations. Journal of the Royal Society, Interface / the Royal Society 3, 15–35.

Gearing, a J., Beckett, P., Christodoulou, M., Churchill, M., Clements, J., Davidson, a H., Drummond, a H., Galloway, W. a, Gilbert, R., Gordon, J.L., 1994. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. Nature 370, 555–557.

Ghosh, A., Pechota, L.V.T.A., Upchurch, G.R.J., Eliason, J.L., 2015. Cross-talk between macrophages, smooth muscle cells, and endothelial cells in response to cigarette smoke: the effects on MMP2 and 9. Molecular and cellular biochemistry 410, 75–84.

Goldberg, G.I., Strongin, A., Collier, I.E., Genrich, L.T., Marmer, B.L., 1992. Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. The Journal of biological chemistry 267, 4583–4591.

Greenwood, J.A., Pallero, M.A., Theibert, A.B., Murphy-Ullrich, J.E., 1998. Thrombospondin signaling of focal adhesion disassembly requires activation of phosphoinositide 3-kinase. The Journal of biological chemistry 273, 1755–1763.

Grimm, T., Chovanová, Z., Muchová, J., Sumegová, K., Liptáková, A., Duracková, Z., Högger, P., 2006. Inhibition of NF-kappaB activation and MMP-9 secretion by plasma of human volunteers after ingestion of maritime pine bark extract (Pycnogenol). Journal of inflammation (London, England) 3, 1.

Hamano, K., Li, T.-S., Takahashi, M., Kobayashi, T., Shirasawa, B., Ito, H., Zempo, N., 2003. Enhanced tumor necrosis factor- alpha expression in small sized abdominal aortic aneurysms. World journal of surgery 27, 476–80.

Han, Y.-P., Nien, Y.-D., Garner, W.L., 2002. Tumor necrosis factor-alpha-induced proteolytic activation of pro-matrix metalloproteinase-9 by human skin is controlled by down-regulating tissue inhibitor of metalloproteinase-1 and mediated by tissue-associated chymotrypsin-like proteinase. The Journal of biological chemistry 277, 27319–27327.

Hanemaaijer, R., Sorsa, T., Konttinen, Y.T., Ding, Y., Sutinen, M., Visser, H., van Hinsbergh, V.W., Helaakoski, T., Kainulainen, T., Ronka, H., Tschesche, H., Salo, T., 1997. Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells. Regulation by tumor necrosis factor-alpha and doxycycline. J Biol Chem 272, 31504–31509.

He, B.J., Joiner, M. a, Singh, M. V, Luczak, E.D., Swaminathan, P.D., Koval, O.M., Kutschke, W., Allamargot, C., Yang, J., Guan, X., Zimmerman, K., Grumbach, I.M., Weiss, R.M., Spitz, D.R., Sigmund, C.D., Blankesteijn, W.M., Heymans, S., Mohler, P.J., Anderson, M.E., 2011. Oxidation of CaMKII determines the cardiotoxic effects of aldosterone. Nature Medicine 17, 1610–1618.

Hellenthal, F. a M.V.I., Buurman, W. a, Wodzig, W.K.W.H., Schurink, G.W.H., 2009. Biomarkers of abdominal aortic aneurysm progression. Part 2: inflammation. Nature reviews. Cardiology 6, 543–52.

Hellenthal, F.A.M.V.I., Geenen, I.L.A., Teijink, J.A.W., Heeneman, S., Schurink, G.W.H., 2009.

Histological features of human abdominal aortic aneurysm are not related to clinical characteristics. Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology 18, 286–93.

Herron, G.S., Unemori, E., Wong, M., Rapp, J.H., Hibbs, M.H., Stoney, R.J., 1991. Connective tissue proteinases and inhibitors in abdominal aortic aneurysms. Involvement of the vasa vasorum in the pathogenesis of aortic aneurysms. Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association 11, 1667–1677.

Hinek, A., Pshezhetsky, A. V., Von Itzstein, M., Starcher, B., 2006. Lysosomal sialidase (Neuraminidase-1) is targeted to the cell surface in a multiprotein complex that facilitates elastic fiber assembly. Journal of Biological Chemistry 281, 3698–3710.

Hingorani, A., Ascher, E., Scheinman, M., Yorkovich, W., DePippo, P., Ladoulis, C.T., Salles-Cunha, S., 1998. The effect of tumor necrosis factor binding protein and interleukin-1 receptor antagonist on the development of abdominal aortic aneurysms in a rat model. Journal of vascular surgery 28, 522–6.

Hipper, A., Isenberg, G., 2000. Cyclic mechanical strain decreases the DNA synthesis of vascular smooth muscle cells. Pflugers Arch 440, 19–27.

Hirose, H., Tilson, M.D., 2001. Abdominal aortic aneurysm as an autoimmune disease. Annals of the New York Academy of Sciences.

Holmes, D.R., Liao, S., Parks, W.C., Thompson, R.W., 1995. Medial neovascularization in abdominal aortic aneurysms: a histopathologic marker of aneurysmal degeneration with pathophysiologic implications. Journal of vascular surgery 21, 761–762.

Holmes, D.R., Lopez-Candales, A., Liao, S., Thompson, R.W., 1996. Smooth muscle cell apoptosis and p53 expression in human abdominal aortic aneurysms. Annals of the New York Academy of Sciences 800, 286–287.

Horstmann, S., Kalb, P., Koziol, J., Gardner, H., Wagner, S., 2003. Profiles of matrix metalloproteinases, their inhibitors, and laminin in stroke patients: influence of different therapies. Stroke; a journal of cerebral circulation 34, 2165–70.

Jones, G., 2011. The Pathohistology of Abdominal Aortic Aneurysm. In: R.T. Grundmann (Ed.), Diagnosis, Screening and Treatment of Abdominal, Thoracoabdominal and Thoracic Aortic Aneurysms. InTech, pp. 53–73.

Josephson, M.A., 2014. Late kidney dysfunction in a kidney transplant recipient. Clinical Journal of the American Society of Nephrology 9, 590–597.

Juvonen, J., Surcel, H.-M.M.H.-M., Satta, J., Teppo, A.-M.M., Bloigu, A., Syrjälä, H., Airaksinen, J., Leinonen, M., Saikku, P., Juvonen, T., 1997. Elevated circulating levels of inflammatory cytokines in patients with abdominal aortic aneurysm. Arteriosclerosis, thrombosis, and vascular biology 17, 2843–2847.

Kagan, H.M., Li, W., 2003. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. Journal of cellular biochemistry 88, 660–672.

Karin, M., 1998. Mitogen-activated protein kinase cascades as regulators of stress responses. Annals of the New York Academy of Sciences 851, 139–46.

Kielty, C.M., Sherratt, M.J., Shuttleworth, C.A., 2002. Elastic fibres. Journal of cell science 115, 2817–2828.

Knäuper, V., Smith, B., Lopez-Otin, C., Murphy, G., 1997. Activation of progelatinase B (proMMP-9) by active collagenase-3 (MMP-13). Eur. J. Biochem. 248, 369–373.

Koch, A.E., Haines, G.K., Rizzo, R.J., Radosevich, J.A., Pope, R.M., Robinson, P.G., Pearce, W.H., 1990. Human abdominal aortic aneurysms. Immunophenotypic analysis suggesting an immune-mediated response. The American journal of pathology 137, 1199–213.

Kol, A., Sukhova, G.K., Lichtman, A.H., Libby, P., 1998. Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor-alpha and matrix metalloproteinase expression. Circulation 98, 300–7.

Kotra, L.P., Zhang, L., Fridman, R., Orlando, R., Mobashery, S., 2002. N-Glycosylation pattern of the zymogenic form of human matrix metalloproteinase-9. Bioorganic chemistry 30, 356–370.

Ladich, E., Yahagi, K., Romero, M.E., Virmani, R., 2016. Vascular diseases: aortitis, aortic aneurysms, and vascular calcification. Cardiovascular Pathology.

Lau, A.C., Duong, T.T., Ito, S., Wilson, G.J., Yeung, R.S.M., 2009. Inhibition of matrix metalloproteinase-9 activity improves coronary outcome in an animal model of Kawasaki disease. Clinical and Experimental Immunology 157, 300–309.

Lau, A.C., Duong, T.T., Ito, S., Yeung, R.S.M., 2008. Matrix metalloproteinase 9 activity leads to elastin breakdown in an animal model of Kawasaki disease. Arthritis and rheumatism 58, 854–863.

Lederle, F. a, Johnson, G.R., Wilson, S.E., 2001. Abdominal aortic aneurysm in women. Journal of vascular surgery 34, 122–6.

Lederle, F.A., Johnson, G.R., Wilson, S.E., Chute, E.P., Hye, R.J., Makaroun, M.S., Barone, G.W., Bandyk, D., Moneta, G.L., Makhoul, R.G., 2000. The aneurysm detection and management study screening program: validation cohort and final results. Aneurysm Detection and Management Veterans Affairs Cooperative Study Investigators. Archives of internal medicine 160, 1425–1430.

Lehoux, S., Castier, Y., Tedgui, A., 2006. Molecular mechanisms of the vascular responses to haemodynamic forces. In: Journal of Internal Medicine. pp. 381–392.

Lehoux, S., Esposito, B., Merval, R., Tedgui, A., 2005. Differential regulation of vascular focal adhesion kinase by steady stretch and pulsatility. Circulation 111, 643–649.

Li, Y.S.J., Haga, J.H., Chien, S., 2005. Molecular basis of the effects of shear stress on vascular endothelial cells. Journal of Biomechanics.

Li, Y.Y., McTiernan, C.F., Feldman, a M., 2000. Interplay of matrix metalloproteinases, tissue inhibitors of metalloproteinases and their regulators in cardiac matrix remodeling. Cardiovascular research 46, 214–224.

Li, Z.Z., Dai, Q.Y., 2012. Pathogenesis of abdominal aortic aneurysms: Role of nicotine and nicotinic acetylcholine receptors. Mediators of Inflammation.

Long, C.S., 2001. The role of interleukin-1 in the failing heart. Heart failure reviews 6, 81–94.

Lopez-Candales, A., Holmes, D.R., Liao, S., Scott, M.J., Wickline, S.A., Thompson, R.W., 1997. Decreased vascular smooth muscle cell density in medial degeneration of human abdominal aortic aneurysms. The American journal of pathology 150, 993–1007.

Lytle, J.R., Yario, T. a, Steitz, J. a, 2007. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5’ UTR as in the 3' UTR. Proceedings of the National Academy of Sciences of the United States of America 104, 9667–9672.

Makrygiannis, G., Courtois, A., Drion, P., Defraigne, J.-O., Kuivaniemi, H., Sakalihasan, N., Ege, L., Danville, B., 2014. Sex Differences in Abdominal Aortic Aneurysm: The Role of Sex Hormones. Annals of Vascular Surgery 28, 1946–1958.

Malek, A.M., Alper, S.L., Izumo, S., 1999. Hemodynamic Shear Stress and Its Role in Atherosclerosis. JAMA 282, 2035–2042.

Marrero, M.B., Paxton, W.G., Schieffer, B., Ling, B.N., Bernstein, K.E., 1996. Angiotensin II signalling events mediated by tyrosine phosphorylation. Cellular Signalling.

Martínez-Ruiz, A., Lamas, S., 2004. S-nitrosylation: A potential new paradigm in signal transduction. Cardiovascular Research.

Maurice, P., Blaise, S., Gayral, S., Debelle, L., Laffargue, M., Hornebeck, W., Duca, L., 2013. Elastin fragmentation and atherosclerosis progression: the elastokine concept. Trends in cardiovascular medicine 23, 211–221.

McCormick, M.L., Gavrila, D., Weintraub, N.L., 2007. Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol 27, 461–469.

McEver, R.P., 2015. Selectins: initiators of leucocyte adhesion and signalling at the vascular wall. Cardiovascular research 107, 331–339.

McFarlane, M.J., 1991. The epidemiologic necropsy for abdominal aortic aneurysm. Jama 265, 2085–2088.

McGregor, J.C., Pollock, J.G., Anton, H.C., 1975. The value of ultrasonography in the diagnosis of abdominal aortic aneurysm. Scottish medical journal 20, 133–137.

Mcmanus, D.D., Freedman, J.E., 2015. MicroRNAs in platelet function and cardiovascular disease. Nature Reviews Cardiology 12, 1–7.

McManus, M.T., Sharp, P. a, 2002. Gene silencing in mammals by small interfering RNAs. Nature reviews. Genetics 3, 737–47.

Merk, D.R., Chin, J.T., Dake, B.A., Maegdefessel, L., Miller, M.O., Kimura, N., Tsao, P.S., Iosef, C., Berry, G.J., Mohr, F.W., Spin, J.M., Alvira, C.M., Robbins, R.C., Fischbein, M.P., 2012. MiR-29b participates in early aneurysm development in Marfan syndrome. Circulation Research 110, 312–324.

Miller, F.J., Sharp, W.J., Fang, X., Oberley, L.W., Oberley, T.D., Weintraub, N.L., 2002. Oxidative stress in human abdominal aortic aneurysms: A potential mediator of aneurysmal remodeling. Arteriosclerosis, Thrombosis, and Vascular Biology 22, 560–565.

Moehle, C.W., Bhamidipati, C.M., Alexander, M.R., Mehta, G.S., Irvine, J.N., Salmon, M., Upchurch, G.R., Kron, I.L., Owens, G.K., Ailawadi, G., 2011. Bone marrow-derived MCP1 required for experimental aortic aneurysm formation and smooth muscle phenotypic modulation. Journal of Thoracic and Cardiovascular Surgery 142, 1567–1574.

Murthy, S., Ryan, A.J., Carter, A.B., 2012. SP-1 regulation of MMP-9 expression requires Ser586 in the PEST domain. The Biochemical journal 445, 229–36.

Nagase, H., Visse, R., Murphy, G., 2006. Structure and function of matrix metalloproteinases and TIMPs. Cardiovascular Research.

Nanda, S., Sharma, S.G., Longo, S., 2009. Molecular targets and abdominal aortic aneurysms. Recent patents on cardiovascular drug discovery 4, 150–9.

Newman, K.M., Jean-Claude, J., Li, H., Ramey, W.G., Tilson, M.D., 1994. Cytokines that activate proteolysis are increased in abdominal aortic aneurysms. Circulation.

Nguyen, M., Arkell, J., Jackson, C.J., 1998. Active and tissue inhibitor of matrix metalloproteinase-free gelatinase B accumulates within human microvascular endothelial vesicles. The Journal of biological chemistry 273, 5400–5404.

Nordon, I.M., Hinchliffe, R.J., Loftus, I.M., Thompson, M.M., 2011. Pathophysiology and epidemiology of abdominal aortic aneurysms. Nature reviews. Cardiology 8, 92–102.

O'Callaghan, C.J., Williams, B., 2000. Mechanical Strain-Induced Extracellular Matrix Production by Human Vascular Smooth Muscle Cells : Role of TGF- 1. Hypertension 36, 319–324.

O'Farrell, T.J., Pourmotabbed, T., 1998. The fibronectin-like domain is required for the type V and XI collagenolytic activity of gelatinase B. Archives of biochemistry and biophysics 354, 24–30.

O'Leary, S.A., Kavanagh, E.G., Grace, P.A., McGloughlin, T.M., Doyle, B.J., 2014. The biaxial mechanical behaviour of abdominal aortic aneurysm intraluminal thrombus: classification of morphology and the determination of layer and region specific properties. Journal of biomechanics 47, 1430–1437.

Ogata, Y., Enghild, J.J., Nagase, H., 1992. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. Journal of Biological Chemistry 267, 3581–3584.

Okamoto, T., Akaike, T., Sawa, T., Miyamoto, Y., Van der Vliet, A., Maeda, H., 2001. Activation of Matrix Metalloproteinases by Peroxynitrite-induced Protein S-Glutathiolation via Disulfide S-Oxide Formation. Journal of Biological Chemistry 276, 29596–29602.

Oleszak, E.L., Chang, J.R., Friedman, H., Katsetos, C.D., Platsoucas, C.D., 2004. Theiler's Virus Infection: A Model for Multiple Sclerosis. Clinical Microbiology Reviews.

Opdenakker, G., Van den Steen, P.E., Dubois, B., Nelissen, I., Van Coillie, E., Masure, S., Proost, P., Van Damme, J., 2001. Gelatinase B functions as regulator and effector in leukocyte biology. Journal of Leukocyte Biology 69, 851–9.

Ørom, U.A., Nielsen, F.C., Lund, A.H., 2008. MicroRNA-10a Binds the 5???UTR of Ribosomal Protein mRNAs and Enhances Their Translation. Molecular Cell 30, 460–471.

Ozsvath, K.J., Hirose, H., Xia, S., Tilson, M.D., 1996. Molecular mimicry in human aortic aneurysmal diseases. Annals of the New York Academy of Sciences 800, 288–293.

Palmieri, D., Perego, P., Palombo, D., 2014. Estrogen receptor activation protects against TNF-α-induced endothelial dysfunction. Angiology 65, 17–21.

Papa, S., Zazzeroni, F., Pham, C.G., Bubici, C., Franzoso, G., 2004. Linking JNK signaling to NF-kappaB: a key to survival. Journal of cell science 117, 5197–5208.

Papazafiropoulou, A., Tentolouris, N., 2009. Matrix metalloproteinases and cardiovascular diseases. Hippokratia.

Paravastu, S.C.V., Jayarajasingam, R., Cottam, R., Palfreyman, S.J., Michaels, J.A., Thomas, S.M., 2014. Endovascular repair of abdominal aortic aneurysm. The Cochrane database of systematic reviews CD004178.

Parks, W.C., Wilson, C.L., Lopez-Boado, Y.S., 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nature reviews. Immunology 4, 617–629.

Parodi, J.C., Palmaz, J.C., Barone, H.D., 1991. Transfemoral intraluminal graft implantation for abdominal aortic aneurysms. Annals of Vascular Surgery 5, 491–499.

Patel, A., Fine, B., Sandig, M., Mequanint, K., 2006. Elastin biosynthesis: The missing link in tissue-engineered blood vessels. Cardiovascular research 71, 40–49.

Pepper, M.S., 2001. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arteriosclerosis, thrombosis, and vascular biology 21, 1104–1117.

Pereira, A.M.M., Strasberg-Rieber, M., Rieber, M., 2005. Invasion-associated MMP-2 and MMP-9 are up-regulated intracellularly in concert with apoptosis linked to melanoma cell detachment. Clinical and Experimental Metastasis 22, 285–295.

Peshkova, I.O., Schaefer, G., Koltsova, E.K., 2015. Atherosclerosis and Aortic Aneurysm: is inflammation a common denominator? FEBS Journal 283, n/a–n/a.

Petrinec, D., Liao, S., Holmes, D.R., Reilly, J.M., Parks, W.C., Thompson, R.W., 1996. Doxycycline inhibition of aneurysmal degeneration in an elastase-induced rat model of abdominal aortic aneurysm: preservation of aortic elastin associated with suppressed production of 92 kD gelatinase. Journal of vascular surgery 23, 336–346.

Pierce, R. a, Moore, C.H., Arikan, M.C., 2006. Positive transcriptional regulatory element located within exon 1 of elastin gene. American journal of physiology. Lung cellular and molecular physiology 291, L391–9.

Ra, H.J., Parks, W.C., 2007. Control of matrix metalloproteinase catalytic activity. Matrix Biology.

Raffetto, J.D., Khalil, R.A., 2008. Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. Biochemical Pharmacology 75, 346–359.

Raffort, J., Lareyre, F., Clement, M., Mallat, Z., 2016. Micro-RNAs in abdominal aortic aneurysms: insights from animal models and relevance to human disease. Cardiovascular research 110, 165–177.

Ramos-DeSimone, N., Hahn-Dantona, E., Sipley, J., Nagase, H., French, D.L., Quigley, J.P., 1999. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. The Journal of biological chemistry 274, 13066–13076.

Ray, A., Bal, B.S., Ray, B.K., 2005. Transcriptional induction of matrix metalloproteinase-9 in the chondrocyte and synoviocyte cells is regulated via a novel mechanism: evidence for functional cooperation between serum amyloid A-activating factor-1 and AP-1. Journal of immunology (Baltimore, Md. : 1950) 175, 4039–4048.

Rensen, S.S.M., Doevendans, P.A.F.M., van Eys, G.J.J.M., 2007. Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation 15, 100–8.

Resnick, N., Yahav, H., Shay-Salit, A., Shushy, M., Schubert, S., Zilberman, L.C., Wofovitz, E., 2003. Fluid shear stress and the vascular endothelium: for better and for worse. Prog Biophys Mol Biol 81, 177–199.

Rizzoni, D., Rodella, L., Porteri, E., Rezzani, R., Sleiman, I., Paiardi, S., Guelfi, D., De Ciuceis, C., Boari, G.E.M., Bianchi, R., Agabiti-Rosei, E., 2003. Effects of losartan and enalapril at different doses on cardiac and renal interstitial matrix in spontaneously hypertensive rats. Clinical and experimental hypertension (New York, N.Y. : 1993) 25, 427–441.

Roderfeld, M., Graf, J., Giese, B., Salguero-Palacios, R., Tschuschner, A., Müller-Newen, G., Roeb, E., 2007. Latent MMP-9 is bound to TIMP-1 before secretion. In: Biological Chemistry. pp. 1227–1234.

Rodríguez, C., Martínez-González, J., Raposo, B., Alcudia, J.F., Guadall, A., Badimon, L., 2008. Regulation of lysyl oxidase in vascular cells: Lysyl oxidase as a new player in cardiovascular diseases. Cardiovascular Research.

Rothuizen, T.C., Kemp, R., Duijs, J.M.G.J., De Boer, H.C., Bijkerk, R., Van der Veer, E.P., Moroni, L., Van Zonneveld, A.J., Weiss, A.S., Rabelink, T.J., Rotmans, J.I., 2016. Promoting tropoelastin expression in arterial and venous vascular smooth muscle cells and fibroblasts for vascular tissue engineering. Tissue engineering. Part C, Methods.

Rouet-Benzineb, P., Gontero, B., Dreyfus, P., Lafuma, C., 2000. Angiotensin II induces nuclear factor- kappa B activation in cultured neonatal rat cardiomyocytes through protein kinase C signaling pathway. Journal of molecular and cellular cardiology 32, 1767–78.

Ruddy, J.M., Jones, J.A., Spinale, F.G., Ikonomidis, J.S., 2008. Regional heterogeneity within the aorta: Relevance to aneurysm disease. Journal of Thoracic and Cardiovascular Surgery 136, 1123–1130.

Ryer, E.J., Ronning, K.E., Erdman, R., Schworer, C.M., Elmore, J.R., Peeler, T.C., Nevius, C.D., Lillvis, J.H., Garvin, R.P., Franklin, D.P., Kuivaniemi, H., Tromp, G., 2015. The potential role of DNA methylation in abdominal aortic aneurysms. International Journal of Molecular Sciences 16, 11259–11275.

Sakalihasan, N., Limet, R., Defawe, O.D., 2005. Abdominal aortic aneurysm. Lancet.

Salmon, M., Johnston, W.F., Woo, A., Pope, N.H., Su, G., Upchurch, G.R., Owens, G.K., Ailawadi, G., 2013. KLF4 regulates abdominal aortic aneurysm morphology and deletion attenuates aneurysm formation. Circulation 128.

Sarén, P., Welgus, H.G., Kovanen, P.T., 1996. TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. Journal of immunology (Baltimore, Md. : 1950) 157, 4159–4165.

Sato, H., Seiki, M., 1993. Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. Oncogene 8, 395–405.

Satoh, H., Nakamura, M., Satoh, M., Nakajima, T., Izumoto, H., Maesawa, C., Kawazoe, K.,

Masuda, T., Hiramori, K., 2004. Expression and localization of tumour necrosis factor-alpha and its converting enzyme in human abdominal aortic aneurysm. Clinical science (London, England : 1979) 106, 301–6.

Schermerhorn, M.L., O'Malley, A.J., Jhaveri, A., Cotterill, P., Pomposelli, F., Landon, B.E., 2008. Endovascular vs. Open Repair of Abdominal Aortic Aneurysms in the Medicare Population. New England Journal of Medicine 358, 464–474.

Scott, R.A., Wilson, N.M., Ashton, H.A., Kay, D.N., 1995. Influence of screening on the incidence of ruptured abdominal aortic aneurysm: 5-year results of a randomized controlled study. The British journal of surgery 82, 1066–70.

Shah, B.H., Catt, K.J., 2003. A central role of EGF receptor transactivation in angiotensin II - induced cardiac hypertrophy. Trends Pharmacol Sci 24, 239–244.

Singh, K., 2001. Prevalence of and Risk Factors for Abdominal Aortic Aneurysms in a Population-based Study : The Tromso Study. American Journal of Epidemiology 154, 236–244.

Sorsa, T., Salo, T., Koivunen, E., Tyynelä, J., Konttinen, Y.T., Bergmann, U., Tuuttila, A., Niemi, E., Teronen, O., Heikkilä, P., Tschesche, H., Leinonen, J., Osman, S., Stenman, U.H., 1997. Activation of type IV procollagenases by human tumor-associated trypsin- 2. Journal of Biological Chemistry 272, 21067–21074.

Sprague, A.H., Khalil, R.A., 2009. Inflammatory cytokines in vascular dysfunction and vascular disease. Biochemical Pharmacology 78, 539–552.

Stather, P.W., Sidloff, D.A., Dattani, N., Gokani, V.J., Choke, E., Sayers, R.D., Bown, M.J., 2014. Meta-analysis and meta-regression analysis of biomarkers for abdominal aortic aneurysm. British Journal of Surgery.

Sterpetti, A. V, Cucina, A., D'Angelo, L.S., Cardillo, B., Cavallaro, A., 1993. Shear stress modulates the proliferation rate, protein synthesis, and mitogenic activity of arterial smooth muscle cells. Surgery 113, 691–699.

Suárez, Y., Fernández-Hernando, C., Yu, J., Gerber, S.A., Harrison, K.D., Pober, J.S., Iruela-Arispe, M.L., Merkenschlager, M., Sessa, W.C., 2008. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. Proceedings of the National Academy of Sciences of the United States of America 105, 14082–7.

Taheri, F., Bazan, H.E.P., 2007. Platelet-activating factor overturns the transcriptional repressor

disposition of Sp1 in the expression of MMP-9 in human corneal epithelial cells. Investigative ophthalmology & visual science 48, 1931–1941.

Tang, T., Boyle, J.R., Dixon, A.K., Varty, K., 2005. Inflammatory abdominal aortic aneurysms. Eur J Vasc Endovasc Surg 29, 353–362.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., Hearn, J.P., 1995. Isolation of a primate embryonic stem cell line. Proceedings of the National Academy of Sciences of the United States of America 92, 7844–8.

Toghill, B.J., Saratzis, A., Harrison, S.C., Verissimo, A.R., Mallon, E.B., Bown, M.J., 2015. The potential role of DNA methylation in the pathogenesis of abdominal aortic aneurysm. Atherosclerosis.

Trial, J., Rossen, R.D., Rubio, J., Knowlton, A.A., 2004. Inflammation and ischemia: macrophages activated by fibronectin fragments enhance the survival of injured cardiac myocytes. Experimental biology and medicine (Maywood, N.J.) 229, 538–45.

Tsou, J.K., Gower, R.M., Ting, H.J., Schaff, U.Y., Insana, M.F., Passerini, A.G., Simon, S.I., 2008. Spatial regulation of inflammation by human aortic endothelial cells in a linear gradient of shear stress. Microcirculation (New York, N.Y. : 1994) 15, 311–323.

Uria, J.A., Lopez-Otin, C., 2000. Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. Cancer research 60, 4745–4751.

Valen, G., Yan, Z.Q., Hansson, G.K., 2001. Nuclear factor kappa-B and the heart. Journal of the American College of Cardiology 38, 307–14.

Van Den Steen, P.E., Wuyts, A., Husson, S.J., Proost, P., Van Damme, J., Opdenakker, G., 2003. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. European journal of biochemistry 270, 3739–3749.

Visse, R., Nagase, H., 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. Circulation Research.

Vorp, D.A., Mandarino, W.A., Webster, M.W., Gorcsan, J., 1996. Potential influence of intraluminal thrombus on abdominal aortic aneurysm as assessed by a new non-invasive method. Cardiovascular Surgery.

Wagenseil, J.E., Mecham, R.P., 2009. Vascular extracellular matrix and arterial mechanics. Physiol Rev 89, 957–989.

Wang, Q., Ren, J., Morgan, S., Liu, Z., Dou, C., Liu, B., 2014. Monocyte Chemoattractant Protein-1 (MCP-1) regulates macrophage cytotoxicity in abdominal aortic aneurysm. PLoS ONE 9.

Wang, Q., Shu, C., Su, J., Li, X., 2015. A crosstalk triggered by hypoxia and maintained by MCP-1/miR-98/IL-6/p38 regulatory loop between human aortic smooth muscle cells and macrophages leads to aortic smooth muscle cells apoptosis via Stat1 activation. International journal of clinical and experimental pathology 8, 2670–2679.

Wei, Y., Schober, A., Weber, C., 2013. Pathogenic arterial remodeling: the good and bad of microRNAs. American journal of physiology. Heart and circulatory physiology 304, H1050–9.

White, G.H., May, J., Waugh, R.C., Chaufour, X., Yu, W., 1998. Type III and type IV endoleak: toward a complete definition of blood flow in the sac after endoluminal AAA repair. Journal of endovascular surgery : the official journal of the International Society for Endovascular Surgery 5, 305–309.

White, G.H., Yu, W., May, J., Chaufour, X., Stephen, M.S., 1997. Endoleak as a complication of endoluminal grafting of abdominal aortic aneurysms: classification, incidence, diagnosis, and management. Journal of endovascular surgery : the official journal of the International Society for Endovascular Surgery 4, 152–168.

Woessner, J.F., 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J. 5, 2145–2154.

Wolinsky, H., 1970. Comparison of medial growth of human thoracic and abdominal aortas. Circulation research 27, 531–538.

Wolinsky, H., Glagov, S., 1964. Structural Basis for the Static Mechanical Properties of the Aortic Media. Circulation research 14, 400–413.

Wu, C.Y., Hsieh, H.L., Sun, C.C., Yang, C.M., 2009. IL-1β induces MMP-9 expression via a Ca2+-dependent CaMKII/JNK/c-Jun cascade in rat brain astrocytes. GLIA 57, 1775–1789.

Wu, H.T., Sie, S.S., Kuan, T.C., Lin, C.S., 2013. Identifying the regulative role of NF-??B binding sites within promoter region of human matrix metalloproteinase 9 (mmp-9) by TNF-?? induction. Applied Biochemistry and Biotechnology 169, 438–449.

Xia, S., Ozsvath, K., Hirose, H., Tilson, M.D., 1996. Partial amino acid sequence of a novel 40-kDa

human aortic protein, with vitronectin-like, fibrinogen-like, and calcium binding domains: aortic aneurysm-associated protein-40 (AAAP-40) [human MAGP-3, proposed]. Biochemical and biophysical research communications 219, 36–39.

Xiong, W., Knispel, R.A., Dietz, H.C., Ramirez, F., Baxter, B.T., 2008. Doxycycline delays aneurysm rupture in a mouse model of Marfan syndrome. Journal of Vascular Surgery 47, 166–172.

Yabluchanskiy, A., Ma, Y., Iyer, R.P., Hall, M.E., Lindsey, M.L., 2013. Matrix metalloproteinase-9: Many shades of function in cardiovascular disease. Physiology (Bethesda, Md.) 28, 391–403.

Yoshida, Y., Sue, W., Okano, M., Oyama, T., Yamane, T., Mitsumata, M., 1990. The effects of augmented hemodynamic forces on the progression and topography of atherosclerotic plaques. Annals of the New York Academy of Sciences 598, 256–273.

Yoshimura, K., Aoki, H., Ikeda, Y., Fujii, K., Akiyama, N., Furutani, A., Hoshii, Y., Tanaka, N., Ricci, R., Ishihara, T., Esato, K., Hamano, K., Matsuzaki, M., 2005. Regression of abdominal aortic aneurysm by inhibition of c-Jun N-terminal kinase. Nature medicine 11, 1330–1338.

Yu, Q., Stamenkovic, I., 2000. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-?? and promotes tumor invasion and angiogenesis. Genes and Development 14, 163–176.

Zhang, H., Park, Y., Wu, J., Chen, X., Lee, S., Yang, J., Dellsperger, K.C., Zhang, C., 2009. Role of TNF-alpha in vascular dysfunction. Clin Sci (Lond) 116, 219–230.

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